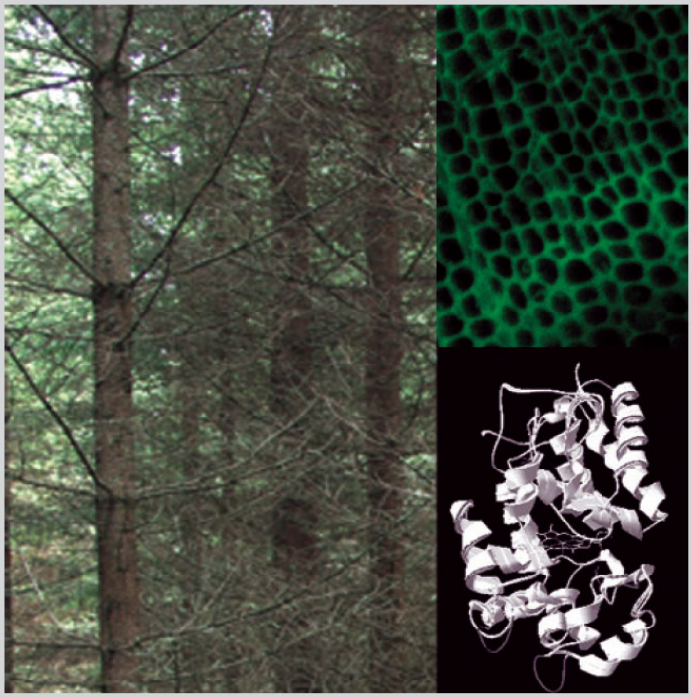

Peroxidases in Lignifying Xylem of Norway Spruce, Scots Pine and Silver Birch

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Front cover: In left: Norway spruce forest (photograph by Pekka Saranpää); In right: Norway spruce xylem and lignin autofluorescence (upper picture, photograph by Ville Koistinen), Norway spruce peroxidase PX1, three dimensional model created using Swiss-Model Server.

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CONTENTS

Abbreviations

ABA, abscisic acid
AldOMT/COMT, hydroxyconiferaldehyde 5-*O*-methyltransferases
4CL, 4-coumarate coenzymeA:ligase
C3H, *p*-coumarate 3-hydroxylase
C4H, cinnamate 4-hydroxylase
CA, coniferyl alcohol
CAD, cinnamyl alcohol dehydrogenase
CAld5H/ F5H, coniferaldehyde 5-hydroxylase
CCoAOMT, caffeoyl coenzyme A 3-*O*-methyltransferase
CCR, cinnamoyl-CoA reductase
CP, C-terminal extension peptide
ctVSD, C-terminal vacuolar sorting determinant
EGFP, enhanced green fluorescent protein
ER, endoplasmic reticulum
FW, fresh weight
HCT, hydroxycinnamoyl CoA: quinate/shikimate hydroxycinnamoyl transferase
IAA, indole-3-acetic acid
IEF, isoelectric focusing
PAL, phenylalanine ammonia lyase
p-CA, *p*-coumaryl alcohol
PCD, programmed cell death
POX, class III plant peroxidase
QTL, quantitative trait locus
RT-PCR, real-time polymerase chain reaction
SA, sinapyl alcohol
SAD, sinapyl alcohol dehydrogenase
SS, secretion signal
TE, tracheary element
VSD, vacuolar sorting determinant

Abstract

Lignin is a complex plant polymer synthesized through co-operation of multiple intracellular and extracellular enzymes. It is deposited to plant cell walls in cells where additional strength or stiffness are needed, such as in tracheary elements (TEs) in xylem, supporting sclerenchymal tissues and at the sites of wounding. Class III peroxidases (POXs) are secreted plant oxidoreductases with implications in many physiological processes such as the polymerization of lignin and suberin and auxin catabolism. POXs are able to oxidize various substrates in the presence of hydrogen peroxide, including lignin monomers, the monolignols, thus enabling monolignol polymerization to lignin by radical coupling.

Trees produce large amounts of lignin in the secondary xylem of stems, branches and roots. In this study, POXs of gymnosperm and angiosperm trees were investigated in order to find POXs which are able to participate in lignin polymerization in the developing secondary xylem i.e. are located at the site of lignin synthesis in tree stems and have the ability to oxidize monolignol substrates. Both in gymnosperm species, Norway spruce (*Picea abies* (L.) Karst.) and Scots pine (*Pinus sylvestris* L.), and in an angiosperm species silver birch (*Betula pendula* Roth) the monolignol oxidizing POX activities originating from multiple POX isoforms were present in lignifying secondary xylem in stems during the period of annual growth. In addition, relatively high POX activities were found from the stems also during late autumn and winter, possibly involved in post-growth lignification observed in earlier studies. Most of the partially purified POXs from Norway spruce and silver birch xylem had the highest oxidation rate with coniferyl alcohol, the main monomer in guaiacyl-lignin in conifers. The only exception was the most anionic POX fraction from silver birch, clearly preferred sinapyl alcohol, the lignin monomer needed in the synthesis of syringyl-guaiacyl lignin in angiosperm trees. However, oxidation of syringyl-type substrates (sinapyl alcohol and syringaldazine) was also seen Norway spruce samples.

Despite the large amount of POX sequences found in databases, most are from angiosperm species the gymnosperms being largely underrepresented. Here, three full-length *pox* cDNAs, *px1*, *px2* and *px3*, were cloned from the developing xylem of the gymnosperm tree species Norway spruce. The predicted proteins coded by these *pox* cDNAs, PX1, PX2 and PX3, showed up to 84% sequence identity to other known POXs. In sequence and phylogenetic analyses, the closest relatives for PX1 protein were lignin-binding POXs from lignin-forming tissue culture of Norway spruce, and for PX2 and PX3 proteins, some POXs from *Pinus* species. *In situ* hybridization experiments showed that transcripts encoding PX1 and PX2 proteins are found in developing tracheids, whereas mRNAs for PX3 were not detected suggesting low transcription level in young trees. According to heterologous expression of *px1* cDNA in *Catharanthus roseus* hairy roots, the protein product of *px1* is a guaiacol-oxidizing POX with an approximate isoelectric point (pI) 10. Similar monolignol oxidizing POXs were found in protein extracts from Norway spruce lignifying xylem.

In accordance to POXs being secreted proteins, the amino acid sequences of PX1, PX2 and PX3 all begin with predicted N-terminal secretion signal (SS) peptides. In addition, PX2 and PX3 contained C-terminal extensions (CPs) which may act as vacuolar sorting determinants (VSDs) in POXs. The subcellular localization of PX1, PX2 and PX3 was studied by transient expression of EGFP-fusions of spruce N-terminal and C-terminal peptides in tobacco protoplasts. It was shown that the N-terminal peptides in PX1, PX2 and PX3 directed EGFP to the endoplasmic reticulum (ER) thus being functional SSs. In tobacco cells expressing fusions of EGFP and N-terminal and C-terminal peptides from PX2, EGFP fluorescence was seen in small vacuole-like and punctate structures. However, in cells expressing fusions of EGFP and these peptides from PX3, EGFP fluorescence was seen as large sheet-like and ER-like structures. Structural

comparison of CPs in PX2, PX3 and in other POXs collected from databases showed that the structure of CP in PX2 was more similar to the CP in vacuolar POX from *A. thaliana* than the CP in PX3.

Hence, the elevated POX activities during secondary growth in gymnosperm and angiosperm tree species studied here arise from multiple coniferyl alcohol- and a few syringyl-oxidizing POX isoforms possibly involved in lignin synthesis. Studies on the cDNA clones of three of these POXs from Norway spruce showed that one of the cloned *poxs*, *px1*, is expressed in lignifying tracheids and codes for a cell wall located protein with similarity to cationic coniferyl alcohol oxidizing POXs. The other Norway spruce *pox*, *px2*, is also expressed in developing tracheids, but codes for a protein which contains a vacuolar localization signal. The third *pox*, *px3*, seems to encode a cell wall located POX protein with low expression level in unstressed spruce seedlings. Further studies on especially the syringyl-specific POXs and transgenic spruces with for example modified *px1* expression levels have potential for revealing the lignin modifying POXs.

List of original publications

This thesis is based on the following original publications which are referred to in text by their Roman numerals. Additional unpublished data will also be presented in the text.

I) Marjamaa K, Lehtonen M [1)], Lundell T [2)], Toikka M [3)], Saranpää P [4)], Fagerstedt KV [5)] (2003) Developmental lignification and seasonal variation in beta-glucosidase and peroxidase activities in xylem of Scots pine, Norway spruce and silver birch. *Tree Physiology* 23, 977-986

II) Marjamaa K, Kukkola E[1)], Lundell T[2)], Karhunen P[3)], Saranpää P[4)], Fagerstedt KV[5)] (2006) Monolignol oxidation by xylem peroxidase isoforms of Norway spruce (*Picea abies*) and silver birch (*Betula pendula*).*Tree Physiology* 26, 605-611

III) Marjamaa K, Hildén K[1)], Kukkola E[2)], Lehtonen M[3)], Holkeri H[4)], Haapaniemi P[5)], Koutaniemi S[6)], Teeri TH [7)], Fagerstedt K[8)], Lundell T[9)] (2006) Cloning, characterization and localization of three novel class III peroxidases in lignifying xylem of Norway spruce (*Picea abies*). *Plant Molecular Biology* 61, 719-732

IV) Marjamaa K and Fagerstedt K[1)]. Function and characterization of C-terminal extensions in Norway spruce class III plant peroxidases. *Manuscript*.

Author's contribution

I. Experimental design with co-authors 2, 4 and 5; Experimental work: enzyme activity measurements with co-author 1, IEF-gel analyses; Interpretation of results with co-authors 4 and 5; Writing of the publication with co-authors 2, 3, 4 and 5, acted as the author in charge.

II. Experimental design with co-authors 2, 4 and 5; Experimental work: purification and analysis of Norway spruce peroxidase fractions; Interpretation of results with co-author 1; Writing of the publication with co-authors 1, 2, 3 and 5, acted as the author in charge.

III. Experimental design with co-authors 1, 6, 7, 8 and 9; Experimental work: sequence analysis of peroxidases with co-authors 1 and 9, phylogenetic analysis, tobacco protoplast transformations and microscopy with co-author 3, *in situ* hybridization probes with co-author 2, protein analysis of *Catharanthus roseus* hairy roots; Interpretation of results with co-authors 1, 2, 8 and 9; Writing of the publication with co-authors 1, 2, 4, 8 and 9, acted as the author in charge.

IV. Experimental design with co-author 1; Experimental work: EGFP constructs, tobacco transformations, sequence analyses and 3-D models; Interpretation of results with co-author 1; Writing of the manuscript with co-author 1, acted as the author in charge.

1. INTRODUCTION

Lignin is a complex aromatic polymer, deposited to plant cell walls where additional strength and stiffness or water impermeability are needed, such as in supporting sclerenchymal structures and water conductive elements in xylem tissue. Lignin polymerization occurs via radical coupling reactions, where enzymes with ability to catalyze oxidation of lignin monomers are needed. For a long time, class III plant peroxidases (POXs) have been implicated to function in lignin polymerization, but despite many intensive studies, the extent of their participation to lignification is still not clear. The following subchapters briefly overview current knowledge on lignin biosynthesis, lignification of xylem cell walls and the class III plant peroxidase superfamily, thus giving background information on the current study concerning the involvement of POXs in lignification of xylem cell walls of gymnosperm and angiosperm trees.

1.1 Lignification of plant cell walls

Lignin is synthesized via co-operation of multiple enzyme activities in cytoplasmic and apoplastic spaces (reviewed by Boerjan et al. 2003). Lignin monomers, mainly the hydroxycinnamyl alcohols coniferyl (CA), sinapyl (SA) and *p*-coumaryl alcohol (*p*-CA), are synthesized in the cytosol and transported to the cell wall where they are linked together to form the lignin polymer. Lignin synthesis in plants can be developmentally regulated or occurs as a response to abiotic or biotic stresses. In developmental lignification lignin is deposited to the plant cell wall during the cell differentiation (reviewed by Marjamaa et al. 2007) whereas lignification associated with defence responses causes fortification of cell walls normally not not lignified and helps for example to restrict the spread of invading pathogens (Menden et al. 2007).

The amount and chemical structure of lignin varies between plant species, different cell types and cell wall layers. Lignin content is typically higher in coniferous wood (25-33%) than in angiosperm wood (20-25%) (Adler 1977). In conifers, lignin is mainly composed of guaiacyl (G) units synthesized from CA, whereas in angiosperms lignin is a co-polymer of SA and CA (syringyl (S) and guaiacyl (G) units) (Nimtz et al. 1981). H-units derived from *p*-CA are present in both angiosperms and gymnosperms in small amounts, but are most abundant in grasses (Nimtz et al. 1981). In addition, relatively high amounts of hydroxycinnamyl acids, most of all *p*-coumarate and ferulate are also found in grass lignins (Grabber et al. 1996, Ralph et al. 1998). At the cellular level, lignin content is higher in areas of cell corner and middle lamella than in secondary cell walls (Agarwal 2006, Gierlinger and Schwanninger 2006). Lignin in vessel cell walls of angiosperm trees contains more G-units than fibre walls and both in angiosperm and gymnosperm trees, H-type lignin is more abundant in the areas of cell corners and middle lamella compared to the other cell

wall layers (Fukushima and Terashima 1991, Grünwald et al. 2002). Lignification is also affected by environmental changes; for example compression wood formed on the lower side of bent stems of conifers is characterized by higher lignin content and higher amounts H-type lignin compared to normal wood (Önnerud and Gellerstedt 2003).

The best characterized example of the process of developmental cell wall lignification is from the cell wall development in xylem cells. The water conducting cells in xylem tissue, tracheary elements (TEs), i.e., tracheids and vessel elements, are hollow dead cells, joined together either with masses of ring pores (tracheids) or with openings at the vertical ends of the cells (vessels). The differentiation of TEs involves deposition of multilamellar secondary cell walls with annular, spiral, reticular, or pitted thickenings and subsequent programmed cell death (PCD) (reviewed by Turner et al. 2007). In addition to lignin, the main components of secondary cell walls are cellulose and hemicelluloses, synthesized by plasma membrane bound cellulose synthase enzyme complexes and Golgi-located glycan synthases and glycosyltransferases, respectively (reviewed by Lerouxel et al. 2006). Lignification of the cell walls begins from cell corners and middle lamellae, proceeding through the secondary cell wall layers following cell wall thickening by carbohydrate deposition (reviewed by Donaldson 2001). The matrix carbohydrate polymers and cellulose microfibrils influence lignin deposition, as demonstrated in *Zinnia elegans* TEs treated with a cellulose synthase inhibitor resulting in dispersed lignification patterns (Taylor et al. 1992). Consequently, in the loose carbohydrate network of the middle lamellae and the primary wall, lignin is spherically formed while in the secondary wall with the strictly oriented cellulose microfibrils lignin forms elongated structures (Donaldson 1994).

In addition to the developmentally regulated lignification, lignification in plants is often induced in stress situations such as wounding (Hawkins and Boudet, 1996) and pathogen infection (Bucciarelli et al. 1998) and after heavy metal (Diaz et al. 2001) or ozone exposure (Cabane et al. 2004). Lignins deposited by plants as stress responses show structural differences to developmental lignins, for example in wheat (*Triticum aestivum*) leaves lignins abnormally rich in S-units are synthesized during defense response (Menden et al. 2007). On the other hand, ozone-treated poplar (*Populus tremula* × *alba*) trees deposit condensed lignins with increased frequency of H-units (Cabane et al. 2004) similar to elicitor-induced lignins in spruce (*Picea abies*) tissue culture (Lange et al. 1995) as well as to early developmental and compression wood lignins (Önnerud and Gellerstedt 2003).

1.1.1 Monolignol biosynthesis

Synthesis of monolignols initiates from the general phenylpropanoid pathway where phenylalanine is converted to *p*-coumaryl CoA via a series of enzymatic reactions, catalyzed by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate coenzymeA:ligase (4CL) (reviewed by Boerjan et al. 2003). *p*-Coumaryl CoA is a precursor for several secondary metabolites in plants, including flavonoids and monolignols.

The schematic view of the enzymatic route for synthesis of monolignols from *p*-coumaryl CoA via aromatic ring hydroxylation, *O*-methylation and conversion of side chain carboxyl to an alcohol group is shown in Figure 1 (modified from Boerjan et al. 2003). This route is supported by the enzymatic activities detected in lignifying tissues and studies with transgenic plants: Significant reduction in total lignin amount has been achieved in transgenic trees where the targets of genetic modification have been genes coding for enzymes involved in the synthesis of apparently all monolignols (e.g. 4CL and CCoAOMT) (Hu et al. 1999, Zhong et al. 2000, Li et al. 2003a), whereas alteration of expression of genes coding for enzymes

specific for sinapyl alcohol synthesis (e.g. AldOMT/COMT, CAld5H/F5H) have had a strong impact on the S/G lignin ratio (Lapierre et al. 1999, Jouanin et al. 2000, Li et al. 2003a). Hydroxycinnamoyl CoA: quinate/shikimate hydroxycinnamoyl transferase (HCT) is located at the branching point of synthesis of monolignols other than *p*-coumaryl alcohol and other products of phenylpropanoid pathway, and recently, it has been shown that the silencing of HCT coding gene causes not only increased proportion of *p*-CA derived lignins (Besseau et al. 2007, Wagner et al. 2007), but also accumulation of flavonoids in plants (Besseau et al. 2007).

On the other hand, there is evidence that the route described in Figure 1 is not always followed. For example, in a recent study on genetically modified alfalfa (*Medigaco sativa*), where several monolignol biosynthetic genes were down-regulated independently, down-regulation of caffeoyl coenzyme A 3-*O*-methyltransferase (CCoAOMT) coding gene did not affect the synthesis of sinapyl alcohol, suggesting that alternative enzymatic routes to same secondary metabolites exist (Chen et al. 2006).

Current knowledge on the regulation of monolignol biosynthesis is limited. There is evidence that genes involved in lignin biosynthesis are controlled at least by the availability of phenolic substrates and carbon resources, hormones and a variety of transcription factors (reviewed by Marjamaa et al. 2007). Feeding loblolly pine (*Pinus taeda*) cell cultures with saturating levels of phenylalanine caused an increase in transcription levels of several genes involved in monolignol biosynthesis and in the amount of coniferyl and *p*-coumaryl alcohol synthesis indicating that the amount of phenylalanine is one of the controlling factors (Anterola et al. 2002). On the other hand, down-regulation of C4H coding gene causes reduced PAL gene expression in transgenic tobacco (*Nicotiana tabacum*) plants, indicating feedback regulation of PAL by cinnamate (Blount et al. 2000). Rogers et al. (2005) have shown that transcription levels of the genes involved in monolignol biosynthesis change according to the circadian rhythm and apparently are induced by increased starch turnover and carbon availability (Rogers et al. 2005).

Aloni et al. (1990) have shown that treating *Coleus blumei* plants with high indole-3-acetic acid (IAA)/low gibberellin GA_3 or low IAA/high GA_3 resulted in increased or decreased S/G lignin ratios in phloem fibers, respectively. Biemelt et al. (2004) have demonstrated that in transgenic tobacco plants with reduced amounts of gibberellin, expression of monolignol biosynthetic genes and the amount of lignin are decreased. Short term feeding of GA_3 to the gibberellin deficient tobacco plants caused an increase in lignin accumulation without transcriptional activation of monolignol biosynthetic genes, suggesting a role for gibberellin also in regulating the transport or polymerization of monolignols (Biemelt et al. 2004). In the *Zinnia elegans* cell culture system, where leaf mesophyll cells trans-differentiate into TEs, supplying of gibberellin in the culture media increases TE lignification while inhibition of endogenous gibberellin synthesis decreases it (Tokunaga et al. 2006).

Quantitative trait locus (QTL) analysis of *Eucalyptus* cDNA microarray data has shown that expression levels of lignin synthesis related genes are regulated by two genetic loci, which in genetic mapping did not co-localize with lignin synthetic genes, suggesting for coordinated control of lignin synthesizing genes by trans-acting factors (Kirst et al. 2004). LIM and MYB type transcription factors can bind to the AC elements found in promoter regions of several genes coding for enzymes in monolignol biosynthesis, and subsequently control the expression of these genes in transgenic plants (Tamagnone 1998; Kawaoka et al. 2000; Kawaoka and Ebinuma 2001, Patzlaff et al. 2003). Genome-wide analysis of lignification related genes in *Arabidopsis thaliana* has shown that in many of the G-type lignin biosynthesis related gene families (PAL, 4CL, HCT, C3H, CCoAOMT, CCR and CAD) at least one member of the

family has AC elements in the promoter region, suggesting a role for AC elements especially in the synthesis of G-type lignin in *A. thaliana*. (Raes et al. 2003). However, over-expression of gene coding for R2R3-MYB transcription factor from *Eucalyptus*, EgMYB2, in transgenic tobacco plants increased expression the genes specific for monolignol synthesis, especially the gene for AldOMT/COMT, and resulted in elevated syringyl-lignin content in the transgenic tobacco plants (Goicoechea et al. 2005). On the other hand, down-regulation of PttMYB21a by antisense expression in transgenic aspen (*Populus tremula*) resulted in increased lignification and transcription of CCoAOMT coding gene, indicating that this MYB transcription factor acts as a transcriptional repressor of lignin biosynthesis (Karpinska et al. 2004). Recently, it has been shown that in double knock-out *A. thaliana* plants deficient in NAC domain transcription factors, NST1/NST3 or SND1/NST1, the lignified secondary cell wall thickenings in stem fibers were suppressed. Transcriptional analysis of the NST1/NST3 and SND1/NST1 inhibited lines revealed reduced expression of genes involved in synthesis of secondary wall components, including genes coding for enzymes involved in lignin biosynthesis (Mitsuda et al. 2007, Zhong et al. 2007). On the other hand, over-expression of *A. thaliana* MYB26 coding gene, increased expression of two NAC-domain transcription factors, NST1 and NST2, and induced ectopic secondary thickening and lignification especially in epidermal tissues of transgenic *A. thaliana* and tobacco plants (Yang et al. 2007).

Altered expression of genes in lignin biosynthesis pathway is a plant response to a variety of external stimuli or stress factors. Ozone and wounding induce genes involved in prechorismate pathway (e.g. phenylalanine synthesis) and monolignol biosynthesis (Cabané et al. 2004, Delessert et al. 2004, Janzik et al. 2005). The phenylpropanoid metabolism and lignin synthetic genes are also induced in pathogen invasion (Adomas et al. 2007, Koutaniemi et al. 2007). On the other hand, in tension wood formed on the upper side of, for example bent branches in angiosperm trees, genes involved in monolignol biosynthesis are down-regulated, leading to reduced lignin content (Andersson-Gunnerås et al. 2006). In aspen tension wood, the MYB transcription factor PttMYB21a with an ability to repress the expression of monolignol biosynthetic genes (Karpinska et al. 2004) was induced suggesting that it acts in down-regulation of lignin biosynthesis in tension wood (Andersson-Gunnerås et al. 2006).

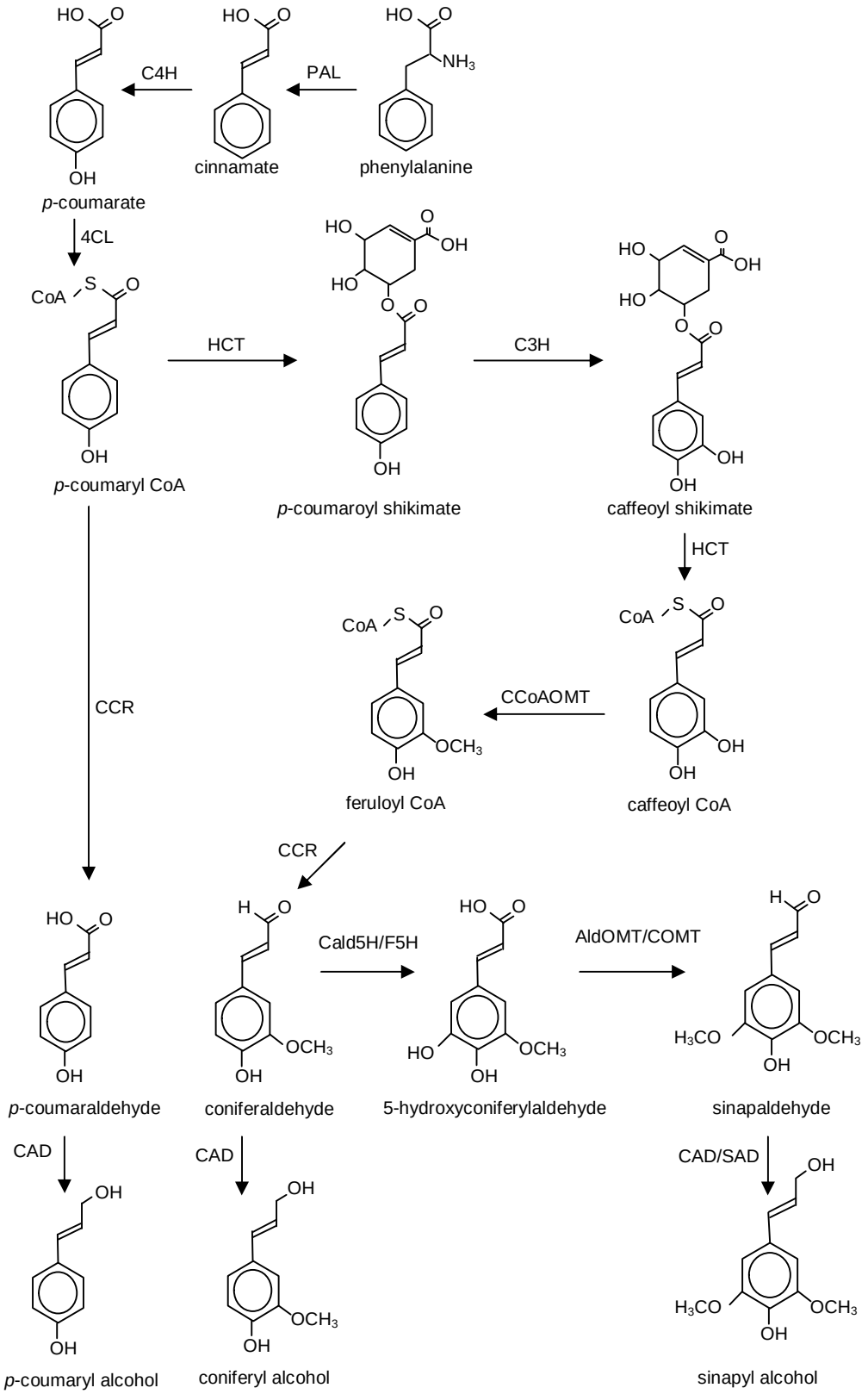


Figure 1. Enzymatic pathway leading to the synthesis of monolignols CA, SA and *p*-CA. See text for abbreviations.

1.1.2 Transport of lignin precursors to the cell wall

Monolignols are found in plants both as free monolignols and as monolignol glucosides. The monolignol glucosides, coniferin, syringin and *p*-coumaryl alcohol glucoside, have been proposed to be either transport forms, intermediates or storage forms of monolignols (Steeves et al. 2001, Tsuji and Fukushima 2004). Coniferin accumulation has been found to correlate spatially and temporally with the beginning of secondary growth in conifers (Freudenberg and Harkin 1963, Savidge 1989). In *Arabidopsis thaliana*, coniferin and syringin accumulation has been observed in light-treated roots (Hemm et al. 2004).

Monolignols are thought to be converted to monolignol glucosides by specific intracellular glucosyltransferases. UDP-glucose dependent glucosyltrasferases catalyzing the glucosylation of sinapyl and coniferyl alcohols have been identified in *A. thaliana* (Lim et al. 2001) and UDP-glucose: coniferyl alcohol glucosyl-transferase activity has been shown to correlate with cambial activity in Jack pine (*Pinus banksiana*) (Savidge and Förster 1998) and Eastern white pine (*Pinus strobus*) (Steeves et al. 2001). Monolignol deglucosylation in turn is thought to occur in the cell wall, at the site of lignin polymerization by specific β-glucosidases. β-glucosidases with the ability to catalyze deglucosylation of monolignols have been identified in Norway spruce (*Picea abies*) (Marcinowsky and Grisebach 1978), in some *Pinus* species (Leinhos et al. 1994, Dharmawardhana et al. 1995) and in *A. thaliana* (Escamilla-Treviño et al. 2006). Samuels et al. (2002) have shown that coniferin β-glucosidase is located to lignifying secondary cell walls of lodgepole pine (*Pinus contorta*). In *A. thaliana*, β-glucosidases *bglu45* and *bglu46* are expressed in organs where lignification is occurring (Escamilla-Tremiño et al. 2006).

Transport of monolignols/monolignol glucosides to the apoplast is thought to occur by Golgi mediated secretion or transport via specific ATP-binding cassette (ABC) transporters. In a high resolution examination of lodgepole pine cambial and xylem sections, Samuels et al. (2002) detected dark staining Golgi vesicles in developing xylem cells in osmiatic samples, suggesting phenolic, possibly monolignol content for them. On the other hand, in global transcript profiling of *A. thaliana* stems, seven genes coding for ABC transporters have shown similar expression profiles to known monolignol biosynthetic genes (Ehlting et al. 2005). The cell wall macroarray analysis of maize (*Zea mays*) brown mid-rib (*bm*) mutants with altered lignin compositions has indicated that in one of the mutant lines, *bm2*, the decreased guaiacyl lignin levels may be due to decreased transcription of one of the ABC transporter genes (Guillaumie et al. 2007).

1.1.3 Monolignol dehydrogenation and polymerization

Lignin polymerization occurs by radical coupling reactions (Freudenberg 1959). Formation of monolignol radicals occurs by dehydrogenation, presumably catalyzed by class III plant peroxidases (POXs), laccases and/or other phenol oxidases. POXs and laccases exist as multigene families in plants (e.g. in *Arabidopsis thaliana* 73 *pox* (Welinder et al. 2002) and 17 laccase genes (McCaig et al. 2005)) and they often have broad substrate spectra and implications to many cellular processes, which makes their functional determination difficult (Mayer and Staples 2002, Passardi et al. 2005).

Laccases are able to oxidize monolignols *in vitro* and laccase genes are expressed in lignifying tissues in plants (Bao et al. 1993, Ranocha et al. 1999, Sato et al. 2001, 2006). Recently, it has been shown that the amount of lignin is decreased in the seed coat of *A. thaliana* plants with a mutation in a laccase gene (Liang et al. 2006). However, although alterations in secondary cell wall structures have been detected in transgenic Western Balsam poplars (*Populus trichocarpa*) with decreased laccase gene expression levels, no reduction in lignification has been observed (Ranocha et al. 2002). Also another type of

oxidase, coniferyl alcohol oxidase, with the ability to oxidize coniferyl alcohol *in vitro* has been purified from lignifying xylem of Sitka spruce (*Picea sitchensis*) (McDougal et al. 1998). Participation of both anionic (Diaz-De-Leon et al. 1993, Christensen et al. 1998, 2001, Østergaard et al. 2000, Li et al. 2003b) and cationic (ElMansouri et al. 1999, Quiroga et al. 2000, Talas-Oğras et al. 2001, Blee et al. 2003, Koutaniemi et al. 2005, Gabaldón et al. 2005) POXs in lignin polymerization has been postulated due to their expression profiles, catalytic properties and impacts of their down-regulation in transgenic plants (discussed in more detail in chapter 3). However, according to present knowledge, there is no evidence that monolignol dehydrogenation would be denoted to a single enzyme and seems more likely that it is done by co-operation of several enzymes or even participation of redox shuttle mechanisms *in vivo* (Önnerud et al. 2002).

While laccases and other oxidases use molecular oxygen in their catalysis, the traditional reaction catalyzed by POXs requires hydrogen peroxide. Many enzymes located in lignifying cell walls, including NADPH oxidases (Ros Barceló et al. 2002), amine oxidase (Moller and McPherson 1998), oxalate oxidases (Caliskan and Cuming 1998) and even POXs themselves (Kawano 2003) are able to catalyze the formation of hydrogen peroxide needed in the peroxidase catalysis. It has been shown that in lignin forming Norway spruce (*Picea abies*) tissue culture (Kärkönen et al. 2002) and differentiating tracheary elements in *Zinnia elegans* cell culture (Karlsson et al. 2005, Gabaldón et al. 2006), availability of hydrogen peroxide is a restricting factor in lignin formation. Furthermore, in *Z. elegans* cultures, hydrogen peroxide accumulates at the sites of secondary cell wall thickening and lignin deposition (Gómez Ros et al. 2006).

In the lignin polymerization process monolignol radicals are linked to the growing polymer via coupling reactions either by carbon-oxygen (ether bond) or carbon-carbon bonds, leading to the formation of a complex network structure. Proceeding of the polymerization requires not only monolignol radicals but also radicals in the existing lignin polymer. The source of radicals in the lignin polymer is not evident. It has been thought that radicals in the polymer could be formed via radical transfer from monolignols or other intermediators, as it has been suggested in the oxidation of sinapyl alcohol, a poor substrate for many peroxidases (Takahama and Oniki 1994, Takahama 1995). However, recently it has been shown that a cationic peroxidase from poplar is able to catalyze the oxidation of polymeric lignols, thus providing a putative direct mechanism for lignin polymer radical formation (Sasaki et al. 2004b).

The most common linkage type found in native lignins is the β-O-4 (β-aryl ether), the others being β-5, β-β, 5-5, β-1 and 5-O-4 (Boerjan et al. 2003). In addition, some lignin substructures such as dibenzodioxocin (Karhunen et al. 1995) have been identified with variant abundances in the lignified cell walls (Kukkola et al. 2004). Studies on native lignins and dehydrogenation polymers of monolignols generated *in vitro* indicate that the abundance of different linkage types and substructures is determined at least by the relative amounts of different monolignols (Boerjan et al. 2003), local monolignol concentrations (Brunow et al. 1998) and the amount of oxidizing enzymes (Mechin et al. 2007) and preformed carbohydrate and lignin (Guan et al. 1997) structures in the wall (Terashima et al. 1995).

It has been suggested that the initiation of lignin polymerization in the middle lamellae and cell corners occurs at specific initiation sites, with which at least extensin-like proteins (Bao et al. 1992), proline-rich proteins (Müsel et al. 1997) and dirigent proteins (Burlat et al. 2001) have been correlated. Dirigent proteins (Latin: *dirigere*, to align or guide) are non-enzymatic cell wall proteins with capability to bind and orient monolignol radicals and thereby promote stereoselective radical coupling (Davin et al. 1997). It has been shown that a dirigent protein guides the coupling of (E)-coniferyl alcohol radicals in formation of lignan (+)pinoresinol, a defence related monolignol dimer (Davin et al. 1997), and it has been postulated that dirigent proteins would direct monolignol

polymerization also in lignin synthesis, thus controlling lignin structure formation (Kim et al. 2002). However, structural variation in native lignins and the flexibility in monomer composition, for example detected in transgenic plants, make the existence of such a tight control mechanism for coupling of monolignols questionable (Boudet 2003).

1.2 Class III plant peroxidases

Peroxidases are enzymes which catalyze oxidoreduction between hydrogen peroxide and reductants. They are found in plants, animals and microbes, and due to their structural and catalytical properties, they are divided into three superfamilies, which can be named as 1) "Animal" peroxidases (although containing glutathione peroxidase, which is also found from plants) 2) catalases from animals, plants, bacteria, fungi and yeast, and 3) "plant peroxidases" from plants, fungi, bacteria and yeast (Hiraga et al. 2001).

The third peroxidase superfamily is divided to three classes. Class I plant peroxidases are intracellular, soluble or membrane-bound peroxidases from plants, bacteria and yeast, such as ascorbate peroxidases, while class II peroxidases are secreted peroxidases from fungi, such as lignin degrading lignin peroxidases and Mn-peroxidases. Class III plant peroxidases (POXs) are secreted plant enzymes found apparently from all land plants but not from unicellular green algae (Passardi et al. 2004). POXs exist as large gene families, for example 73 genes are found in *Arabidopsis thaliana* (Welinder et al. 2002) and 136 in the rice (*Oryza sativa*) genome (Passardi et al. 2004), and they are implicated in various physiological processes vital for plant life from "seed to senescence" (Passardi et al. 2005).

1.2.1 POX structure and catalysis

The structures and catalytic mechanisms are well characterized for several POX variants (Smith and Veitch 1998, Schuller et al. 1996, Gajhede et al. 1997, Østergaard et al. 2000). Class III peroxidases are metalloproteins containing an extractable heme (Fe+ protoporphyrin IX) center and two stabilizing Ca^{2+} -ions, one distal and one proximal to the heme plane. The crystal structures of five of six POXs determined so far show that they have similar active site structures and protein folds, with 13 α-helices held together in compact globular structure (Schuller et al. 1996, Gajhede et al. 1997, Mirza et al. 2000, Østergaard et al. 2000, Henriksen et al. 2001). Structure of barley grain peroxidase BP1 differs from the other structurally determined peroxidases by being inactivated at pH above five, and by having a distorted loop in the structure (Henriksen et al. 1998).

POXs are glycosylated to varying degree, for example cationic horse radish peroxidase HRPC protein structure contains eight N-linked glycans (Welinder et al. 1979), whereas the majority of POXs in *Arabidopsis thaliana* contain one to two putative glycosylation sites (Welinder et al. 2002). Although the POX amino acid sequences identities can be less than 35% within a plant species, several amino acid residues involved in the heme-binding and peroxidase catalysis are well-conserved, as well as the two calcium-binding sites, the S-S-bridge forming cysteines and the buried salt-bridge motif involved in the fold-formation (Welinder et al. 2002).

POX amino acid sequences typically initiate with a well recognizable amino (N)-terminal secretion signal peptide (SS) for transport of the protein into the endoplasmic reticulum (ER), and in the absence of other localization determinants, further secretion to the cell wall (Hiraga et al. 2001). Some POX sequences, like HRPC and barley grain peroxidase BP2, contain carboxyl (C)-terminal extension peptides (CP), which have been associated to vacuolar localization of POXs (Theilade et al. 1993). In HRPC (Welinder 1979) and BP2 (Johansson et al. 1992), these extensions are not found in purified proteins indicating that they are removed during protein maturation. In addition, some POX sequences contain additional N-terminal extensions of unknown function after the secretion signal peptide (Welinder et al. 2002).

In the regular POX catalytic cycle, one equivalent of H_2O_2 is consumed and two equivalents of reducing substrates are oxidized via three redox states of the enzyme (Figure 2, Berglund et al. 2002, Liszkay et al. 2003). POXs typically lack strict specificity for reducing substrates being able to oxidize a wide variety of phenolic compounds, phenolic domains of feruloyated polysaccharides, tyrosine residues of cell wall structural proteins and auxin (reviewed by Hiraga et al. 2001). Additionally, in the presence of superoxide or reducing substrates such as auxin, POXs can catalyze the reduction of hydrogen peroxide or oxygen to ·OH or HOO·, respectively, in the so called hydroxylic cycle (Figure 2, Berglund et al. 2002, Liszkay et al. 2003).

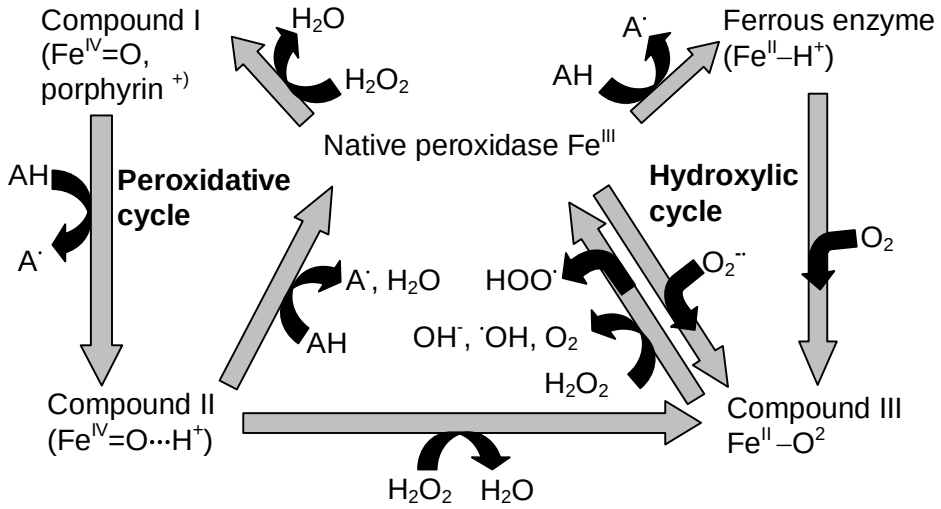


Figure 2. Class III peroxidase catalytic cycles, adapted from Berglund et al. (2002) and Liszkay et al. (2003). AH, reducing substrate.

1.2.2 POX functions

POXs are expressed in plants during various developmental processes and as responses to abiotic and biotic stresses. Most commonly the prediction of their function in different physiological situations comes from the detection of POX gene expression/POX proteins *in situ*, knowledge of their catalytic properties *in vitro* and subsequent structural or biochemical etc. changes occurring putatively by the action of POX enzymes. There are a few studies where down-regulation or up-regulation of POX genes has resulted in detectable physicochemical changes in transgenic plants (see below). However, clear "loss-of-function" -changes in POX deficient plants has not been detected, apparently due to their functional redundancy.

1.2.2.1 POXs in cell wall modification

Monolignol oxidation/dehydrogenation is one of the earliest cellular functions proposed for POXs (Harkin et al. 1973). Transcription of POX genes has been correlated with lignification in many plants species and POX isoforms with the capability to oxidize monolignol substrates have been purified from lignifying tissues (∅stergaard et al. 2000, Quiroga et al. 2000, Christensen et al. 2001, Gabaldón et al. 2005, Sato et al. 2006). It has been shown that alterations in *pox* gene expression can have an impact on lignification patterns: over-expression of the gene coding for a cationic POX under 35S promoter caused ectopic lignification in transgenic tomato (*Lycopersicon esculentum*) (El Mansouri et al. 1999) plants, whereas down-regulation of the genes coding for a cationic POX in transgenic tobacco (*Nicotiana tabacum*) (Blee et

al. 2003) and an anionic POX in transgenic aspen (*Populus tremula*) (Li et al. 2003b) resulted in up to 50% and 20% reduction in lignin amounts, respectively. However, no single POX responsible for monolignol dehydrogenation in lignin synthesis has been found.

Suberin is a structurally variant cell wall polymer containing chemically distinct aromatic and aliphatic domains. The aliphatic domain is composed of, for example α-hydroxyacids (mainly 18-hydroxyoctadec-9-enoic acid) and α,ω-diacids (mainly octadec-9-ene-1,18-dioic acid), whereas the aromatic domain is a polymer of hydroxycinnamic acids and their derivatives (reviewed by Franke and Schreiber 2007). Suberin deposition restricts the flow of solutes and gases via the cell walls, and it occurs in exodermis and endodermis of roots and as a response to wounding (Franke and Schreiber 2007). POXs are able to oxidize hydroxycinnamic acid monomers of suberin thus enabling their polymerization (Arrieta-Baez and Stark 2006) and POXs are also found in suberin synthesizing tissues of tomato (Mohan et al. 1993, Quiroga et al. 2000), potato (*Solanum tuberosum*) (Bernards et al. 1999) and musk melon (*Cucumis melo*) (Keren-Keiserman et al. 2004). On the other hand, down-regulation of the gene coding for an anionic tomato POX correlated with suberization caused no phenotypic changes in transgenic tomato plants (Sherf et al. 1993).

In addition to apparent involvement of POXs in lignification and suberization, POXs seem to be able to control the cell wall properties in different developmental phases and in stress responses by cross-linking the structural non-enzymatic proteins such as extensins, by catalyzing the formation of diferulic acid linkages between polysaccharide bound lignins or ferulic acid residues in polysaccharides (Fry 2004) and by production of hydroxyl radical with the ability to cleave cell wall polysaccharides (Schweikert et al. 2000). Cross-linking of extensins by isodityrosins and cell wall polymers by diferulic acid bridges is associated with the cessation of cell elongation (Brownleader et al. 2000) and cell wall fortification in defense events (Deepak et al. 2007). Cross-linking of extensins by POXs occurs apparently at motifs containing Tyr and Lys residues (Schnabelrauch et al. 1996, Held et al. 2007). POXs with the ability to cross-link extensins have been characterized from tomato (Schnabelrauch et al. 1996), lupin (*Lupinus albus*) (Price et al. 2003) and grapevine (*Vitis vinifera*) (Jackson et al. 2001) whereas POX participation in ferulic acid cross-linking and in growth cessation has been proposed for example in stems of maritime pine (*Pinus pinaster*) (Sánchez et al. 1996) and leaf blades of tall fescue (*Festuca arundinacea*) (MacAdam and Grabber 2002). On the other hand, cell elongation requires cell wall loosening and thus changes in the polysaccharide and protein networks. This is thought to be mediated for example by polysaccharide modifying enzymes such as xyloglucan endotransglucosylase-hydrolases (Cosgrove 2003). It is known that hydroxyl radicals are able to cleave cell wall polysaccharides pectin and xyloglucan *in vitro*, thus providing one mechanism for cell wall loosening (Fry 1998). Interestingly, Schweikert et al. (2000) have shown that the production of hydroxyl radicals acting in polysaccharide scission can be catalyzed by POX.

1.2.2.2 Auxin metabolism and other signaling

Auxins are plant hormones involved in the regulation of many physiological processes including xylem formation and cell elongation (reviewed by Teale et al. 2006). POXs are able to oxidize IAA both via the regular peroxidative cycle and molecular oxygen consuming hydroxylic cycle (Figure 2, Kawano 2003). Structural similarities corresponding to auxin-binding site of other auxin-binding proteins are found from POXs (Savitsky et al. 1999). In transgenic tobacco plants over-expressing anionic POX which oxidizes IAA *in vitro*, the reduced lateral root formation was suggested to be caused by enhanced auxin degradation by POX (Gazaryan and Lagrimini 1996, Lagrimini et al. 1997). On the other hand, the hydrogen

peroxide-independent oxidation of IAA and salisylic acid (SA) radicals generated by POX oxidation, can mediate hydrogen peroxide production, which in turn can act as signaling molecule for example in defense responses (Kawano 2003).

1.2.2.3 Other POX functions

POXs have been reported to function also in the synthesis of other plant secondary metabolites, than the macromolecules lignin and suberin. A basic POX isolated from *Catharanthus roseus* leaves was able to act as alfa-3',4'-anhydrovinblastine (AVLB) synthase apparently by oxidizing vindoline and catharanthine and thus allowing their dimerization to AVLB, a monoterpenoid indole alkaloid (Sottomayor and Ros Barcelo 2003). A POX purified from leaves of *Bupleurum salifocium* showed specific activity for caffeic acid and ferulic acid thus catalyzing the synthesis of possibly defence-related dimers (Frías et al. 1991). POXs are also able to oxidize anthocyanins, such as pelargonin, resulting for example in precipitation via polymerization or browning of these pigments (Wang et al. 2004). There are also indications that POXs are able to detoxify heavy metals and other toxic molecules. Cadmium is detoxified in the waterlily *Nymphaea* by trapping it into peroxidase generated phenolic polymers as Ca-Cd crystals (Lavid et al. 2001a, 2001b), while degradation of the toxic pesticide 2,4-dichlorophenol in turnip (*Brassica napus*) hairy root cultures was probably due to POX activity (Agostini et al. 2003). In addition, POXs have been associated with plant protection against UV-radiation: over-expression of the gene encoding an anionic POX caused increased UV tolerance in transgenic tobacco plants (Jansen et al. 2001).

1.2.3 Regulation of POX expression and catalysis

Expression of *pox* genes is typically found in many plant organs and developmental phases. In real time (RT)-PCR analysis of spatial and temporal expression of 33 *Arabidopsis thaliana poxs* it was shown that 16 of these *poxs* are expressed in growing *A. thaliana* plants constantly (Welinder et al. 2002). Almost all the *pox* genes were expressed in roots, 13 of them being expressed also in all the other organs i.e. rosettes, stems, cauline leaves and flower buds (Welinder et al. 2002). Nine of the *poxs* were transcribed only in roots, whereas only one of these *poxs* was specific for stems, rosettes and cauline leaves (Welinder et al. 2002). However, detailed information on the basis of developmental regulation of *pox* genes is scarce. In maritime pine (*Pinus pinaster*) roots *pox* gene expression was induced for example by auxins and ethylene (Charvet-Candela et al. 2002) whereas the promoter for the gene for a tobacco anionic POX was strongly suppressed by auxin (Klotz and Lagrimini 1999). The promoter of the gene encoding a cationic POX in Korean radish (*Raphanus sativus*) was activated by gibberellic acid but suppressed by abscisic acid (ABA) (Lee and Kim, 1998). This promoter was also activated by low ratio of cytokinin to auxin (Kim et al. 2004).

Stress responses often include *pox* gene expression. It has been shown that some wound-inducible *pox*s are induced by jasmonic acid and/or ethylene, which are associated to wound-signaling (Ishige et al. 1993, Sasaki et al. 2004a). On the other hand, expression of a wound-inducible *tpoxN1* in tobacco was not induced by jasmonic acid or ethylene (Sasaki et al. 2002), but its promoter is activated by binding of AP2/ERF type transcription factor to the vascular system-specific and wound-responsive cis-element (VWRE) in the promoter (Sasaki et al. 2007). Promoter of wound-inducible *pox* from horseradish (*Armoracia rusticana*) *prxC2* is induced by binding of NtLIM1 transcription factor to the AC-elements in the promoter (Kaothien et al. 2001). On the other hand, promoter for an oxidative stress inducible anionic *pox* from sweet potato (*Ipomea batatas*) contained several oxidative stress responsive elements and was induced in transgenic tobacco plants by hydrogen peroxide, wounding and UV-light (Kim et al. 2003). The gene for a cationic POX

in *A. thaliana* was induced by cold-treatment, dehydration, ABA and salt stress and negatively regulated by light (Llorente et al. 2002).

Its is known that POX activity can be controlled by external factors such as pH (Henriksen et al. 1998) and naturally by the availability of hydrogen peroxide and reducing substrates. In a proteomic analysis of maize cell suspension cultures, elicitor treatment caused rapid dephosphorylation of some extracellular POXs (Chivasa et al. 2005). The dephosphorylation of POXs may allow regulation of the activity of these POXs at post-translational level. In addition, spatial distribution of POXs in cell wall may control their action. Some POXs can bind to the plasma membrane (Mika and Lüthje, 2003) or cell wall macromolecules such as pectin (Carpin et al. 2001) and lignin-like polymers (McDougal et al. 2001b, Warinowski, pers. com.), which may in part control the function of these POXs.

1.3 Aims of the present study

Trees form a great portion of the biomass on Earth and contain large amounts of lignin in the secondary xylem formed during radial growth. Trees provide raw material for construction and pulp and paper industry and are in addition a significant source of energy. All these forms of utilization are affected by the lignin composition of trees. Hence, gaining information on factors affecting lignin synthesis in trees evokes both a scientific and an economic interest.

In the present work, properties of POXs in lignifying stem xylem of Finnish gymnosperm and angiosperm tree species, Norway spruce (*Picea abies* (L.) Karst.), Scots pine (*Pinus sylvestris* L.) and silver birch (*Betula pendula* Roth) were studied in order to find POXs with a capability to contribute to the final stage of lignin synthesis, the dehydrogenative polymerization of monolignols. Revealing the function of different POXs in lignification helps us to understand the impact of their action on the composition of lignin in trees and may give us valuable tools for controlling wood properties.

In general, POXs which participate in lignin synthesis in the developing xylem have to be able to oxidize monolignols and must be located in the lignifying cell wall of xylem cells. Here, POXs which would meet these criteria were searched from the developing xylem of tree species in study with several experimental approaches. First, the temporal relationship between lignification of xylem cells and presence of POX activities and isoforms were studied in Norway spruce, Scots pine and silver birch (I, II). Second, several POX isoforms were partially purified from stem xylem of Norway spruce and silver birch and their monolignol oxidation capability was examined (II). Third, three cDNAs coding for POXs were cloned from differentiating stem xylem of Norway spruce, their translation products were examined *in silico* and to some extent *in vivo* and their site of action was studied at the cellular and tissue specific level (III, IV).

2. MATERIALS AND METHODS

2.1. Xylem samples from forest grown Norway spruce, Scots pine and silver birch trees

Stem samples were obtained from trees grown at Ruotsinkylä Experimental Station of the Finnish Forest Research Institute in southern Finland (lat. 60°21′ N, long. 24°51′ E; (incorrect coordinates in article II)). In articles I and II, the stands were naturally regenerated, and so trees represented wide genetic variation. In article III, clonal trees (E8504) were used. Samples for different purposes were collected and handled as described in articles I, II and III.

2.2 Methods described in the articles

Method	Article
Protein extractions and quantifications	I, II
POX activity assays	I, II
β-glucosidase activity assays	I
Student's T-test	I
Coniferin synthesis	I
Isoelectric focusing gels (IEF)	I, II
Histologiacl preparations	I
Purification of POXs	II
Production and analysis of dehydrogenation polymers (DHP)	II
RNA extraction and cloning and sequencing of *pox* cDNAs	III
Preparation of green fluorescent protein (GFP)-constructs	III, IV
Preparation, transformation and detection of tobacco protoplasts	III, IV
In situ hybridization	III
Expression and examination of spruce POX in *Catharanthus roseus* hairy roots	III
Structural and phylogenetic analysis of POXs	III, IV

3. RESULTS AND DISCUSSION

3.1 POX and β-glucosidase activities and lignification in stem xylem of trees

POX activity is commonly found in lignifying tissues of trees (Harkin et al. 1973, Polle et al. 1994, 1997, Christensen et al. 1998, 2001). Here, the relationship between POX activities and lignification of xylem cells walls was studied in two gymnosperm tree species, Norway spruce (*Picea abies* (L.) Karst) and Scots pine (*Pinus sylvestris* L.), and one angiosperm species, silver birch (*Betula pendula* Roth) (I). For this study, stem samples were collected from five tree individuals of each tree species over the growing season, from mid-winter to late autumn, for histological preparations and enzyme activity measurements (I).

3.1.1 Radial growth and lignification of stem xylem

In the trunks of trees, cell divisions in cambial layer produce several layers of new xylem cells during every growing season. Vast portion of these cells differentiate into tracheids (in conifers) and vessel elements or structural fibers (in broad-leaf trees), during which they form thick lignified secondary cell walls and finally perform programmed cell death (PCD). A subset of xylem cells develop into parenchyma cells, which in rays may remain alive for several years (Nakaba et al. 2006).

Timing of radial growth and lignification of the stem xylem in the conifers Norway spruce and Scots pine and the broad-leaf tree species silver birch was determined by examining safranin-Alcian blue stained cryo-sections from stem samples collected over the growing season (I). Safranin-Alcian blue treatment is a common method for staining plant derived histological preparations (VonAufsess 1973, Srebotnik and Messner 1994). Alcian blue binds to cell wall polysaccharides giving them blue coloration, and safranin stains phenolic groups in lignin, thereby enabling distinction between trachery elements (tracheids and vessel elements, TEs) and fibers of different developmental stage due to the level of their cell wall lignification.

Radial growth in stems of the conifers Norway spruce and Scots pine began in May and was seen as several layers of unlignified or partially lignified developing tracheids in the cryo-sections, and continued until August-September when all the tracheids in the sections showed thick cell walls with intense safranin staining (I). Growth of trees is environmentally controlled by temperature and day length (Nitch 1957, Junttila 1986, Antonova and Stasova 1997, Rossi et al. 2006, Gyllenstrand et al. 2007), the temperature being especially important for cambial reactivation after dormancy (Druart et al. 2007) and the day-length to the maximal growth rate (Rossi et al. 2006).

Thin-walled developing vessel elements and fibers were first seen in stem sections of the broad-leaf tree species silver birch in samples collected in early June (I). The later initiation

of xylem radial growth in the deciduous birch compared to conifers could be caused by the lack of photosynthesizing leaves at the beginning of the growing season and concurrent direction of energy reservoirs mainly to leaf development (Piispanen and Saranpää 2001). In samples from early August, the cell walls of all vessels and fibers in birch sections were thick and fully stained with safranin (I) indicating cessation of xylem growth, and the beginning of accumulation of storage carbohydrates and proteins in preparation to dormancy (Clausen and Aspel 1991, Piispanen and Saranpää 2001, Druart et al. 2007).

Lignification in cell walls of the developing tracheids detected by safranin staining was visible already in the first samples where xylem growth was observed in all tree species in the study (I). This is in agreement with *Zinnia elegans* cell culture, where differentiating tracheary elements (TEs) deposit lignified secondary cell wall thickenings and perform PCD within 72 h of culture (Obara and Fukuda 2005). Safranin staining was first seen in the regions of cell corners and middle lamellae, at the sites of initiation of lignification (Donaldson 2001). By the end of September in conifers and in early August in birch, cell walls of all the new tracheids were lignified, characterized by intense safranin staining of thickened secondary cell walls (I). However, it has been shown that lignification of cell walls in latewood tracheids in conifers continues until the following spring (Donaldson 1987, Polle et al. 1997), and even during the development of the first new earlywood tracheids (Christiernin 2006). Increasing lignin amount in the xylem cell walls during winter months or early spring could not be detected with the experimental setting used in this study, but it can be assumed that this kind of lignification is maintained by preformed enzymes and monolignol remnants in the mature cell walls, or even through monolignol feeding from ray parenchyma cells. Namely, it has been observed in TE-forming *Z. elegans* cell cultures that the monolignols that polymerize into lignin in developing TE cell walls are supplied by not only the TEs but also by the parenchymatic cells via the culture medium (Hosokawa et al. 2001). Furthermore, while genes coding for lignin polymerizing enzymes (laccases and a peroxidase) were specifically expressed in lignifying TEs, the monolignol biosynthetic genes were expressed both in lignifying TEs and in non-lignifying parenchymal cells (Demura et al. 2002).

3.1.2 POX activities in lignifying stem xylem

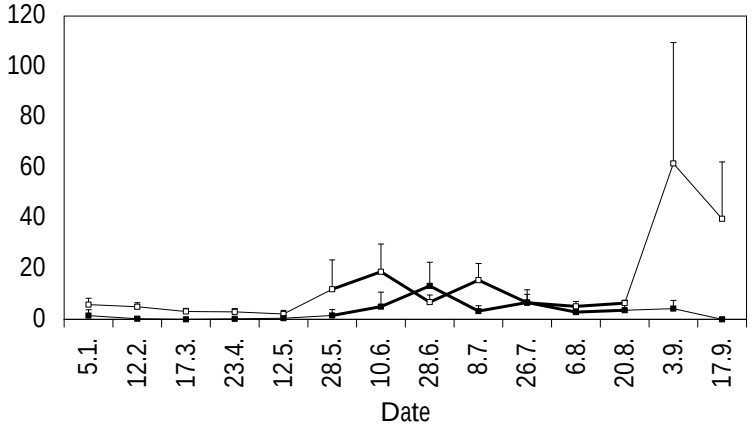
For studying the relationship of POX activity and xylem lignification described above, proteins were extracted from the outermost xylem for enzyme activity measurements (I). Xylem samples were extracted with high salt buffer in order to collect soluble and ionically cell-wall-bound proteins, which apparently comprise most of the POX population in stem xylem of these tree species (I, Marjamaa et al. 2004).

POX activities in xylem protein extracts collected through the growing season were measured with guaicol, a small hydroxyphenol commonly used in the POX activity measurements, and coniferyl alcohol (CA), a natural lignin monomer in both conifers and broad-leaf trees (I). POX activities in katals (mol/s) were calculated both per fresh weight (FW) and per protein amount in order to get a comprehensive picture of the variation of POX activity in the tissue. Since relatively high level of variation was observed in enzyme activities between different tree individuals, statistical significance of differences in POX activities at different time points was estimated using Student's T-test. Variation in POX activities showed similar trends with both guaiacol and coniferyl alcohol substrates, as observed earlier for example by Polle et al. (1994) by studying POXs in Norway spruce needles. However, some slight differences between CA and guaiacol oxidation patterns were seen especially in Norway spruce and silver birch samples, possibly reflecting different affinities of different POXs for these substrates (I).

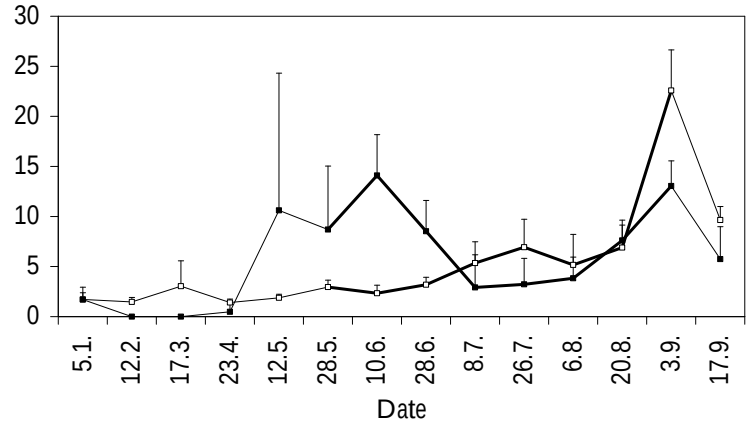
POX activities calculated per FW (Figure 3) showed the closest relations to xylem growth and lignin synthesis in conifers, where POX activities increased significantly in May at the beginning of radial growth (I). In Scots pine POX activities continued to increase through the summer, as the amount of new xylem cells in the samples increased (I, Figure 3). However, in Norway spruce samples POX activities began to decrease gradually towards late August prior to the cessation of growth (I, Figure 3). Similarly, Polle et al. (1997) have noticed that although soluble POX activity in shoot axes of Norway spruce initially increased with tissue lignification, the POX activity decreased before the cessation of lignin accumulation. They suggested that this may be caused by the inactivation of POXs by their phenolic substrates, lower extractability of POXs due to covalent binding to cell walls or enzyme degradation (Polle et al. 1997). On the other hand, the decrease in POX activity may also be caused by cessation of some other POX-related cellular process than lignin synthesis. Also in silver birch samples, an increase in POX activities per FW was detected after initiation of xylem growth and coinciding with rapid lignification of new xylem cells (I). However, silver birch POX activities showed also a peak in the spring prior to the initiation of growth, presumably arising from pre-existing ray cells (I, Figure 3).

A remarkable increase in POX activities was detected in all the tree species in late-August-September (I, Figure 3). In addition, relatively high values for POX activities were measured in the xylem samples collected during winter. Winter-time POX activities were especially high when calculated per total protein content, meaning that the proportion of POXs in protein population of the xylem extracts increased during winter apparently due to the absence of other proteins related to xylem growth and differentiation. High POX activities in trees during autumn and winter period has been reported earlier for branches of *Populus* × *euamericana*, xylem sap of beech (*Fagus sylvatica*), stems of Scots pine (Fagerstedt et al. 1998) and needles and shoot axes of Norway spruce (Polle and Glavac 1993, Polle et al. 1994, 1997). Presence of POX activities in the xylem during winter period in January and February suggests that active POXs remain in the cell walls of mature xylem cells and/or vacuoles of ray cells after trees become dormant for the cold season. Active POXs in the mature cell walls of xylem cells, if supplied with monolignols and hydrogen peroxide, could be involved in winter and spring-time lignification detected in conifer tracheids (Donaldson 1987, Polle et al. 1997, Christiernin 2006).

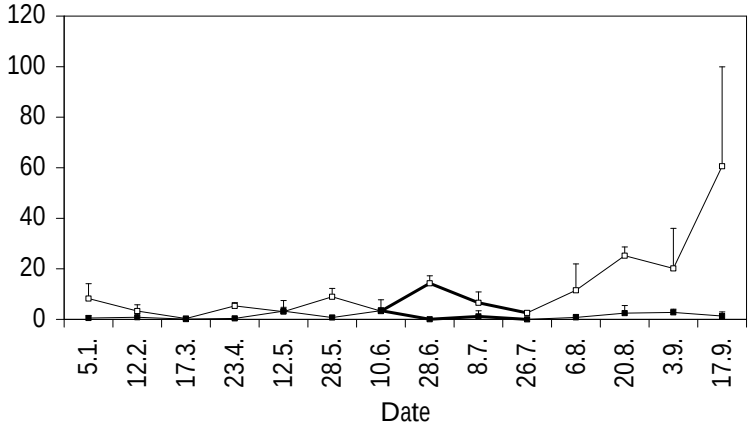
Thus, elevated POX activities were found from lignifying xylem of all the gymnosperm and angiosperm species in this study. Although part of the detected POX activity is probably involved in other processes than lignification, lignin deposition is a major process in xylem development and could require high POX activities.



A)



B)



C)

Figure 3. POX and β-glucosidase activities in xylem samples of Norway spruce (*Picea abies*) (A), Scots pine (*Pinus sylvestris*) (B) and silver birch (*Betula pendula*) (C) measured with CA (open symbols, nkat/g FW) and coniferin (closed symbols, fkat/g FW), respectively. Thick lines represent the time period when secondary growth and lignification was observed in the cryo-sections.

3.1.3. β-glucosidase activities in lignifying stem xylem

In addition to POXs and oxidases, β-glucosidases have been associated with the last stages of lignin monomer biosynthesis in the cell walls, i.e., the release of monolignols from their possible glucosidic conjugates. β-Glucosidase activities were also measured in the xylem protein extracts of Norway spruce, Scots pine and silver birch in order to compare patterns of POX and β-glucosidase activities (I). β-glucosidase activities were measured with the synthetic *p*-nitrophenyl-β-*O*-D-glucopyranoside (4-NPG) and the natural substrate coniferin, the glycosylated form of coniferyl alcohol.

In vitro β-glucosidase activities were much lower than POX activities in xylem extracts (I). Further, coniferin β-glucosidase activities were markedly lower than 4-NPG hydrolyzing activities suggesting the presence of coniferin specific β-glucosidase in xylem extracts. Such enzymes have been found earlier in the xylem of Norway spruce and some pine species (Marcinowsky and Grisebach 1978, Leinhos et al. 1994, Dharmawardhana et al. 1995). In Norway spruce coniferin β-glucosidase activity gradually began to increase in May at the time of the initiation of growth and reached a maximum in late June and decreased suddenly at the end of the growth period in early September (I, Figure 3A). In pine samples, coniferin β-glucosidase activity began to increase already before the initiation of growth, decreased significantly in June-July in the middle of the growth period and showed again a significant peak in early September, coinciding with a peak in POX activity (I, Figure 3B). In birch samples, coniferin β-glucosidase activities were rather low and showed no association with xylem growth (I, Figure 3C). In contrast to the POX activities, coniferin β-glucosidase activities were low during winter months in all the tree species in this study (I, Figure 3).

As the xylem samples were extracted here with a high salt buffer, the enzyme activities measured originate from the soluble and ionically bound cell wall proteins. However, a portion of the proteins may not have been solubilized by the high salt treatment, due to for example covalent bonds between the proteins and other cell wall components. Marcinowsky and Grisebach (1978) discovered that coniferin β-glucosidase activity in seedlings of Norway spruce was not completely released by 0.6 M NaCl. Thus, some coniferin hydrolyzing β-glucosidase activity may have remained in the xylem powder after extraction with high salt buffer and is not visible in this study.

Hence, increase in coniferin specific β-glucosidase activity possibly related to the intiation of secondary growth was seen in conifers. However, no relationship between coniferin β-glucosidase activity and growth and lignification was seen in the birch samples, questioning the importance of coniferin in the synthesis of birch developmental lignins.

3.2 Xylem POX isoforms and their substrate preferences

POXs are encoded by a multigene family and typically several POX genes are expressed in any given plant organ (Welinder et al. 2002). Consequently, multiple POX isoforms with variant isoelectric points (pI) are typically found in stem xylem extracts of trees (Tsutsumi et al. 1998, Christensen et al. 1998, McDougal 2001a). Although POXs can often oxidize a wide spectrum of phenolic substrates, it has been observed that they may discriminate for example between guaiacyl- and syringyl-type substrates (∅stergaard et al. 2000).

3.2.1. Seasonal variations in POX isoform patterns

Associations between different POX isoforms and lignification in stems of Norway spruce, Scots pine and silver birch were studied here by running isoelectric focusing gels (IEF) from

the xylem protein extracts from samples collected over the growing season (I).

In IEF gels from Norway spruce and Scots pine samples, several guaiacol-oxidizing POX isoforms with isoelectric points (pI) ranging from 3.5-10 were detected throughout the study period (I). The increase at the beginning of xylem growth in Scots pine seems to be associated with an increase in cationic POX activity band intensity, whereas in Norway spruce both anionic and cationic POXs showed more intense staining after the beginning of xylem growth. Participation of cationic POXs to xylem lignification has been suggested for example in Norway spruce needles, stems of white poplar (*Populus alba*) and *Zinnia elegans* cell culture (Polle et al. 1994, Gabaldon et al. 2005, Sasaki et al. 2004, 2006). However, in birch the most dominant POX isoforms during xylem growth and lignification were anionic POXs with pI 3.5 and 3.8 (I, Figure 10: incorrect figure legend, should be "Peroxidase isoenzymes in silver birch xylem…"). Anionic POXs have been associated also with lignification for example in stems of Western Balsam poplar (*Populus trichocarpa*) (Christensen et al. 1998, 2001), and down-regulation of an anionic POX has been found to result in reduced lignin content in transgenic aspen (*Populus tremula*) (Li et al. 2003b). Thus, relevance of cationic and anionic POXs in lignin synthesis may be species dependent and/or multiple enzymes with different pIs may participate in the polymerization of lignin in plants.

3.2.2. Substrate preferences of POX isoforms in the xylem of Norway spruce and silver birch

POX isoforms were partially purified from larger scale stem xylem samples from Norway spruce and silver birch to reveal their possible preferences for monolignol substrates (II). In the case of Norway spruce, washing of the xylem powder with acetone prior to protein extraction was needed to diminish the amount of interfering phenolics and extractives in the samples (II, Marjamaa et al. 2004). A positive effect of washing with organic solvent to POX purification from trees has been previously observed by Fürtmüller et al. (1996). However, such treatment was not necessary for POX purification from silver birch (II).

Preparative IEF was the most efficient way to separate cationic and anionic POX isoforms of Norway spruce, since the cationic spruce POX isoforms showed apparently unspecific binding to the column chromatography matrices (II, Marjamaa et al. 2004). In contrast, separation of birch POX isoforms was obtained by a sole anion exchange chromatography step (II).

Five POX fractions from Norway spruce and three fractions from silver birch were obtained and their abilities to oxidize monolignol substrates *in vitro* was studied (II). Enzyme activity measurements with coniferyl (CA), sinapyl (SA) and *p*-coumaryl (*p*-CA) alcohol showed that all the cationic, neutral and anionic POX fractions from Norway spruce had the highest oxidation rates with CA, the main monomer in spruce lignin (II). Similar results have been obtained earlier with POXs from gymnosperm tree species (Tsutsumi et al. 1998, McDougal 2001a). In contrast, the most anionic POX fraction from silver birch showed clearly the highest oxidation rate with SA, the lignin monomer needed for the synthesis of guaiacyl-syringyl lignin in birches (II).

SA is a poor substrate for many POXs, and it has been suggested even that SA dehydrogenation by POXs is mediated by other phenolic radicals (Takahama and Oniki 1994, Takahama 1995). The origin of this SA discrimination has been searched from the structure of substrate binding site of POXs by docking of monolignol substrates and ferulic acid into the X-ray structure of *A. thaliana* peroxidase ATP A2 (∅stergaard et al. 2000). According to this, docking of ferulic acid and CA gave identical hydrophobic interactions with the enzyme involving amino acid residues P69, I138, P139, S140, R175 and V178. Docking of *p*-CA showed fewer interactions (namely P69, II138, P139 and S140), but docking of SA in the same orientation as the other substrates was unsuccessful due to the

overlapping of I138 and P139 with the additional methoxyl group of SA (∅stergaard et al. 2000). This substrate discrimination of ATP A2 to sinapyl compounds was confirmed experimentally by kinetic analyses with CA, ferulic acid, coumaric acid and sinapic acid by Nielsen et al. (2001). Since P139 is conserved in the POX superfamily, ∅stergaard et al. (2000) proposed that binding of SA is structurally hindered in all family members.

However, SA oxidizing POXs have been purified from tomato (*Lycopersicon esculentum*) (Quiroga et al. 2000), *Z. elegans* (Gabaldón et al. 2005) and poplar (*Populus alba*) cell culture (Ayoama et al. 2002). Furthermore, the SA oxidizing POX from poplar was able to oxidize even larger substrates, synthetic lignin polymers (Sasaki et al. 2004) On the basis of this Sasaki et al. (2004) suggested the existence of an additional substrate oxidation site on the surface of the enzyme, as in fungal lignin peroxidase (Johjima et al. 1999) and in cytochrome *c* peroxidase (Miller et al. 1995).

Recently, Gómez Ros et al. (2007) attempted to identify the structural motifs that characterize POXs with the ability to catalyze oxidation of syringyl moieties. However, although a cationic peroxidase PAPX5 isolated from a tissue culture of Norway spruce showed all the structural motifs detected by Gómez Ros et al. (2007), it was not able to oxidize SA *in vitro* (Koutaniemi et al. 2005). Taken together, making conclusive determinations on substrate specificities of various POXs based on structural modeling seem to be difficult.

Substrate oxidation capabilities of different POXs in the xylem of Norway spruce and silver birch was also studied by comparing activity stained IEF gels using guaiacol or syringaldazine (SYR), an artificial POX substrate resembling sinapyl alcohol (II). SYR-oxidizing POXs have been associated with lignin synthesis in trees as SYR-oxidizing POX activity localizes to the developing xylem tissue in gymnosperm and angiosperm tree species (Harkin and Obst 1973, Christensen et al. 2001).

While guaiacol was oxidized by several POX isoforms in Norway spruce and silver birch xylem extracts, SYR stained only a few POX isoforms (II). In the xylem samples from Norway spruce, the only POX isoforms stained with SYR were cationic with pI 9, whereas in birch samples the SYR-oxidizing isoforms were anionic, with pI 3.6, 4.5 and 4.9.

The most anionic SYR-oxidizing POX in birch extracts may correspond to the anionic SYR-oxidizing POX purified from Western Balsam poplar (*Populus trichocarpa*) (Christensen et al. 1998). The SYR-oxidizing POX isoform was found also in the sinapyl alcohol-preferring POX fraction purified from the birch xylem, suggesting that this is the POX with preference for sinapyl compounds (II). The cationic SYR-oxidizing POX from Norway spruce was also found in the most cationic POX fraction purified from spruce xylem (II). Although this cationic POX fraction oxidized SA with higher rate than other purified spruce fractions, the best substrate for the fraction was clearly CA. However, this finding may be merely caused by the low amount of the SYR-oxidizing POX isoform in the purified fraction (II).

Although syringyl units are rare in lignin of many conifer species, Gómez Ros et al. (2007) demonstrated that they are found in significant amounts in some gymnosperm lignins for example in the conifer *Tetraclinis articulata* and gnetopsid *Ephedra viridis*. Further, SA-oxidizing POX activity was found in all gymnosperm species in their study, independent from the lignin composition, suggesting that SA-oxidizing POXs are remnants from ancient S/G lignin synthesizing gymnosperms (Gómez Ros et al. 2007).

On the other hand, the ability of poplar (*Populus alba*) SA oxidizing POX to oxidize polymeric substrates suggests a role for this type of POXs in the radical formation in the lignin polymer (Sasaki et al. 2004b). Such POX activity could be necessary in the synthesis of both guaiacyl (spruce) and guaiacyl-syringyl (birch) type lignins.

3.3 Spruce POX structure and expression

Although significant amounts of POX sequences are available in databases for molecular level analyses, most of the sequences originate from angiosperm plant species (Duroux and Welinder 2003). In PeroxiBase (http://peroxidase.isb-sib.ch/), a peroxidase specific database (Bakalovic et al. 2006), altogether 41 full length POX sequences from gymnosperm species were found, compared to for example 117 POXs from Western balsam poplar (*Populus trichocarpa*), a single angiosperm tree species (with full genomic sequence available). In order to obtain molecular information on the POXs in the developing xylem in gymnosperm tree species, *pox* cDNAs were cloned from the RNA pool extracted from developing xylem of Norway spruce using reverse transcription and PCR amplification with primers designed for conserved regions in known *pox*s (III).

3.3.1. Structure and characterization of Norway spruce POXs

Three full-length *pox* cDNAs clones, *px1*, *px2* and *px3*, from developing xylem of Norway spruce, were obtained eventually. The predicted amino acid sequences (PX1, PX2 and PX3) of these POXs contained all the conserved amino acid residues needed for POX structure and catalysis, were less than 60% identical at amino acid level and in a BLAST (http://www.ncbi.nml.nih.gov) search showed over 70% identity to previously identified POXs in databases (III). Similarly to known POXs, the PX1, PX2 and PX3 proteins had predicted pI:s from ~5-9.5 and molecular masses around 35 kDa.

Currently, eight other full-length Norway spruce POXs can be found from databases. One them is the pathogen induced POX SPI2 isolated from infected roots of Norway spruce (Fossdal et al. 2001) and the other seven originate from the lignin-forming Norway spruce tissue culture (Koutaniemi et al. 2005, Koutaniemi et al. 2007). Additionally, eight other POXs sequences were found from the EST database created from the differentiating xylem of Norway spruce (Koutaniemi et al. 2007). Quantitative real time-PCR (RT-PCR) analysis by Koutaniemi et al. (2007) showed that at least eleven of these altogether 19 spruce *pox*s are expressed in lignifying xylem of mature trees, corresponding well to the amount of POX isoforms detected in Norway spruce xylem extracts (II).

In an expression analysis of lignification-related genes in *Arabidopsis thaliana*, several genes coding for POXs had expression patterns similar to those of monolignol biosynthetic genes (Ehlting et al. 2005). The differences in expression levels of different *pox* genes in lignifying tissues can be interpreted as indicators of the importance of the corresponding POX proteins in the lignification process. In a quantitative RT-PCR analysis of *px1*, *px2* and *px3* showed moderate expression levels in the differentiating xylem of mature trees, but were not in the group of most highly expressed *pox*s (Koutaniemi et al. 2007). However, it has been observed that POXs with even relatively low level of gene expression can be abundant at the protein level, indicating high stability of these POX proteins and/or mRNAs (Christensen et al. 2001).

3.3.2 Phylogenetic analyses of POX sequences

Phylogenetic analyses from the Clustal aligned amino acid sequences of PX1, PX2 and PX3, and various other POXs found from the databases, were performed in order to find POXs with similar primary structure, arising from the same phylogenetic origin, putatively evolved for the same cellular function (III).

In the Neighbour-joining tree containing POXs from Norway spruce, some other tree species and various herbaceous species, PX1 clustered for example with SPI2, the pathogen induced POX from Norway spruce (Fossdal et al. 2001), POXs isolated from lignin-forming Norway spruce tissue culture (Koutaniemi et al. 2005), lignin associated POX from

Townsville stylo (*Stylosanthes humilis*) (Talas-Ogras et al. 2001) and cationic POX from peanut (*Arachis hypogaea*) (Schuller et al. 1996) (III). However, PX2 and PX3 fell into different cluster grouping together with PSYP1, a short root specific POX from Scots pine (*Pinus sylvestris*) (Tarkka et al. 2000), AtP4 from *Arabidopsis thaliana* (Welinder et al. 2002), BP1 from barley (*Hordeum vulgare*) (Henriksen et al. 1998) and some other POXs from herbaceous species (III).

In a large scale phylogenetic study of POX gene family in plants by Duroux and Welinder (2003), 20 phylogenetic groups within POXs in dicot plants were identified. Most of the monocot POXs did not cluster in same groups with dicot POXs but formed five additional monocot specific groups, indicating that distinct structures have evolved in POXs after dicot-monocot separation (Duroux and Welinder 2003). Tree construction was not performed with gymnosperm POXs in that work due to limited amount of sequences available at the time of the study. However, by sequence comparison they suggested grouping of PSYP1 (Tarkka et al. 2001) from Scots pine with *A. thaliana* POX AtP4 and emergence of this POX group before divergence of gymnosperms and angiosperms (Duroux and Welinder 2003). In the Neighbour-Joining tree in the present study (III), AtP4 clustered with PSYP1, PX3 and PX2, as suggested by Duroux and Welinder (2003). Duroux and Welinder (2003) also identified AtPRX18 as the most similar *A. thaliana* POX to pathogenesis induced POX from spruce, SPI2 (Fossdal et al. 2001). According to this, SPI2 and the paralogous PX1 would not be related to POXs from peanut or Townsvile stylo observed in the present study, but with POXs from for example tomato and potato (Duroux and Welinder 2003).

In order to define POX clusters within gymnoperm species, an unrooted Fitch-Margoliash tree was built from the Clustal-aligned full-length POXs from gymnosperm species in the PeroxiBase (Figure 4). In these trees, PX1 clustered with SPI2 and lignin-binding POXs isolated from spruce tissue culture (Koutaniemi et al. 2007), whereas PX2 and PX3 clustered both with PSYP1 and some other POXs from pine species (Figure 4).

PX1, PX2 and PX3 protein sequences were compared with POXs from the same phylogenetic groups in gymnosperm POX tree by pairwise alignments using the Needle software in order to define the degree of similarity within the clusters (Table 1). This showed that while the spruce POXs described here, PX1, PX2 and PX3, were less than 60% identical to each other, PX1 was 71.6% identical to SPI2 and 84.8% identical to Norway spruce POXs PX16 and PX17, originally isolated as lignin-bound isoforms from Norway spruce lignin forming tissue culture (Koutaniemi et al. 2007). Comparison of PX2 and PX3 with the POXs from pine species in the same branch showed that while PX3 was up to 81.5% identical to POXs from pine species in the branch, identity between PX2 and any other POXs in the study was always less that 61%.

POXs in same phylogenetic groups may have evolved for similar biological functions (Duroux and Welinder 2003). Phylogenetic relative of PX1, SPI2, was originally isolated from pathogen infected spruce roots. SPI2 over-expression under 35S promoter caused reduced flexibility, deeper-red phloroglucinol-HCl staining (lignin stain with highest reactivity to coniferaldehyde) and increased amount of aldehyde end-groups in lignin β-O-4 G units in transgenic tobacco plants (Elfstrand et al. 2002). There is evidence that the lignin-binding POXs PX16 and PX17 from Norway spruce tissue culture are able to produce structurally native lignin-resembling dehydrogenation polymers (DHPs) from CA *in vitro* (pers. com. with T. Warinowski). Among the phylogenetic relatives of PX3, PSYP1 is specifically expressed in short roots of Scots pine, and the authors discussed that it may be associated with modification of cell walls or with restriction of cell expansion by auxin catabolism in these organs (Tarkka et al. 2000).

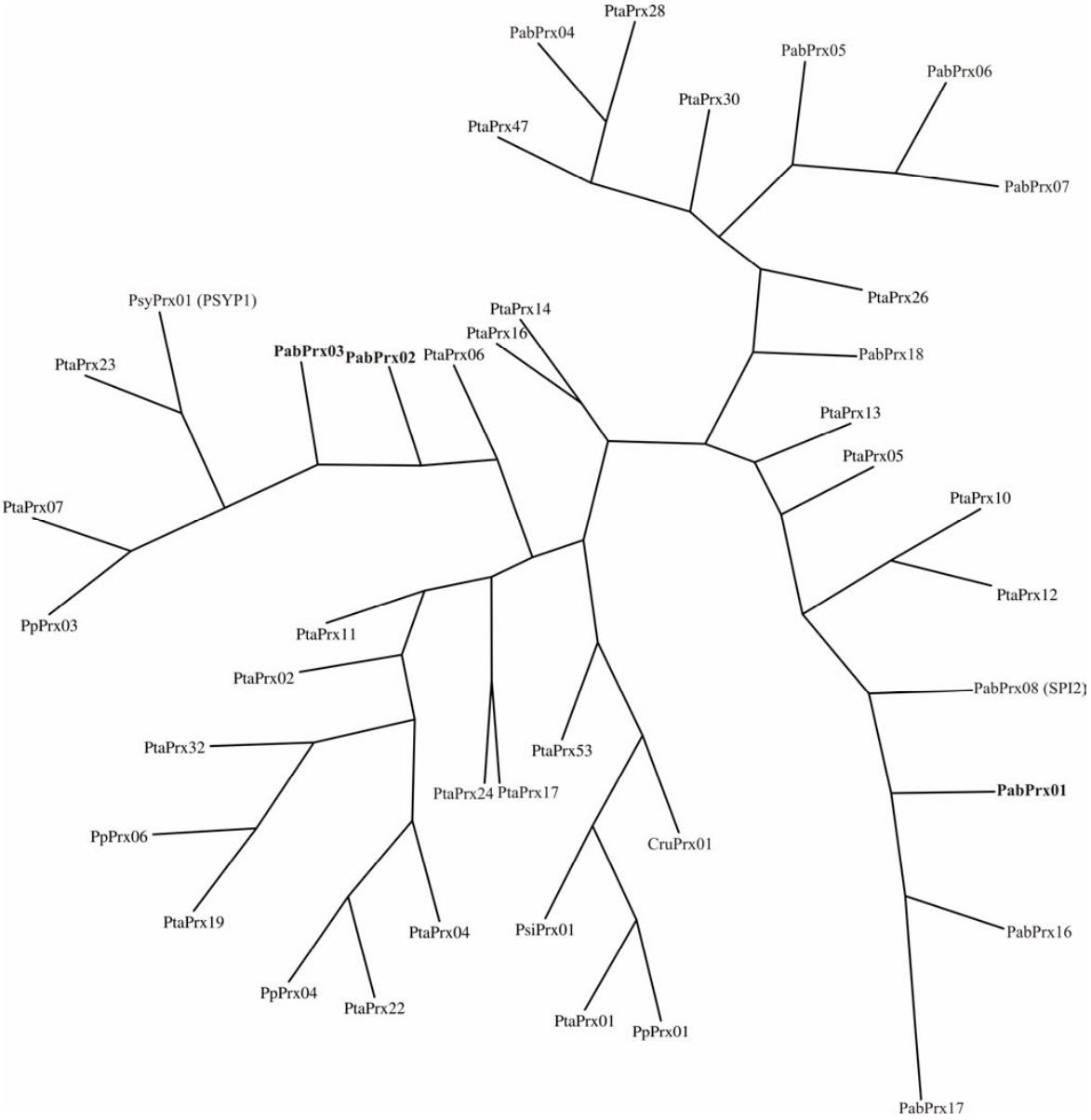


Figure 4. Fitch-Margoliash tree visualizing phylogenetic relationships between gymnosperm POXs retrieved from PeroxiBase. Norway spruce peroxidases in this study are marked in bold. Pta, *Pinus taeda*; Pp, *Pinus pinaster*; Psy, *Pinus sylvestris*; Psi, *Picea sitchensis*; Pab, *Picea abies*; Cru, *Cycas rumphii*; Prx, POX.

Table 1. Amino acid identities (%) between PX1-3 and the other gymnosperm POXs in their phylogenetic groups. POXs in the same tree branch are marked with bold numbers. Pab, *Picea abies*; Psy, *Pinus sylvestris*; Pp, *Pinus pinaster*; Pta, *Pinus taeda.*

	PabPrx08	PabPrx16	PabPrx17	PsyPrx01	PpPrx3	PtaPrx23	PtaPrx7	PtaPrx6
PabPrx1 (PX1)	**71.6**	**84.8**	**84.8**	40.2	41.1	40.2	40.1	39.3
PabPrx2 (PX2)	37.0	39.2	39.7	**56.2**	**59.3**	**56.1**	**60.6**	**53.5**
PabPrx3 (PX3)	37.6	40.9	41.5	**79.1**	**79.3**	**78.7**	**81.5**	**54.6**

3.3.3 Spatial distribution of *px1*, *px2* and *px3* expression in stem tissues of Norway spruce

The RNA pools from Norways spruce used in *pox* cloning originate from two cell types, differentiating tracheids and ray parenchyma cells, both of which may have POX enzyme activity *in situ* (Fagerstedt et al. 1998, Christensen et al. 2001). In order to find the primary expression sites of *px1*, *px2* and *px3*, *in situ* hybridization experiments were performed with stem sections from Norway spruce seedlings (III). The experiments showed that both *px1* and *px2* transcripts were found in developing tracheids but not in ray cells (III). Tracheid specific expression pattern of *px1* and *px2* is different from that of lignin polymer oxidizing *pox* in white poplar (*Populus alba*), since this POX protein was found in fiber walls and ray cells, but not inside the developing fibers, suggesting that it is synthesized in ray cells (Sasaki et al. 2006).

However, *px3* transcripts were not detected in stems of Norway spruce seedlings at all indicating a low expression level. It may be that *px3* function is related to physiology of more mature trees and/or it is induced by other than developmental cues (III). POX gene expression has been frequently observed to be induced in stress situations such as wounding or pathogen invasion (Sasaki et al. 2004, Bae et al. 2006). In fact, RT-PCR experiments by Koutaniemi et al. (2007) have shown that both *px3* and *px2* are induced in the phloem of mature spruce after fungal infection. Furthermore, both *px2* and *px3* are also induced in compression wood xylem in young trees, indicating a general stress inducible expression pattern for these *poxs* (Koutaniemi et al. 2007).

3.3.4 Heterologous expression of Norway spruce POXs in *Catharanthus roseus* hairy roots

In order to examine the protein products of *px1*, *px2* and *px3*, an approach was taken to create transgenic *Catharanthus roseus* hairy roots expressing the Norway spruce (*Picea abies*) *poxs* under the 35S promoter. Here, transgenic hairy root lines expressing *px1* and *px2* were obtained, while all the lines transformed with *px3* suffered from early death. For identification of protein products of *px1* and *px2*, proteins were extracted from root cultures and analyzed in IEF gels with guaiacol staining (III). In the protein extracts from *px1*-expressing hairy root lines, an additional cationic POX isoform was observed in IEF gels compared to the wild type lines (III). In IEF gels from protein extracts from *px2* expressing hairy root line, no *px2* protein product could be distinguished possibly due to the high amount of native POXs with similar pI (estimated pI 8.37) in the samples (Data not shown).

The isoelectric point of the putative *px1* protein product was approximately ten, which is higher than the estimated pI of PX1 (III). In POXs, this may be due to binding of heme and two calcium ions in correct POX fold formation (Welinder et al. 2002). The intensity of the putative *px1* product increased when proteins were extracted with high salt containing buffer, indicating that this POX is ionically bound to the cell wall (III). A POX isoform with a similar pI to the putative *px1* product was found in xylem protein extracts of Norway spruce during developmental lignification (I) and in the partially purified cationic POX fraction with ability to oxidize coniferyl alcohol (II).

3.4 Subcellular localization of POXs

POXs have been found from cell walls and vacuoles in plants, and the subcellular localization of POXs naturally has a great impact on the variety of physiological processes they can be involved in. Heggie et al. (2005) showed that the heterologously expressed HRPC with or without vacuolar localization signal caused different kind of phenotypes in transgenic tobacco (*Nicotiana tabacum*) plants, such as decreased root development in the plants expressing HRPC without vacuolar signal compared to the

unchanged root development in the vacuolar HRPC expressing plants. The cell wall located putative functions of POXs include for example construction and modification of cell wall components and auxin catabolism, whereas vacuolar POXs may be involved for example in the synthesis of defense related substances (Hiraga et al. 2001, Passardi et al. 2005).

Endoplasmic reticulum (ER) is the first cellular compartment in the protein secretion route. Proteins are directed to ER by specific secretion signal peptides (SS) in the amino terminus of the protein chain, after which the signal peptide is removed (Alberts et al. 2002). The SSs, characterized by a positively charged n-region, a hydrophobic h-region and a neutral but polar c-region prior to the signal peptidase cleavage site, can be recognized from the protein amino acid sequence by the neural network based software SignalP (Nielsen et al. 1997). All the three Norway spruce POX protein sequences PX1, PX2 and PX3, as well as all the other full-length POXs isolated from Norway spruce (Fossdal et al. 2001, Koutaniemi et al. 2005, Warinowski et al. unpublished), begin with predicted N-terminal SSs, recognized by TargetP (Emmanuelsson et al. 2000) and SignalP (Nielsen et al. 1997) softwares. It is expected that in the absence of additional localization signals the proteins are secreted by the default pathway to the cell wall.

At least two kinds of vacuoles, lytic vacuoles and storage vacuoles have been found in plants. Vacuolar sorting determinants (VSDs) have been identified in both the amino (N) and carboxyl (C) terminus of plant proteins (reviewed by Robinson et al. 2005). The N-terminal VSDs found in plant proteins have a specific consensus sequence motif (e.g. NPIR), whereas no specific motifs responsible for vacuolar sorting have been identified in many C-terminal VSDs. The non-sequence specific vacuolar sorting determinants (ctVSDs) are typically 10-20 amino acids long and highly hydrophobic (Neuhaus and Rogers, 1998). Although no consensus sequence has been found in these localization signals, there is evidence that at least the accessibility of the terminal amino acids affects the sorting efficiency of ctVSDs, since blocking the C-terminus by addition of glycines or glycosylation caused secretion of otherwise vacuolar barley lectin to the cell wall (Dombrowski et al. 1993).

No sequence-specific vacuolar sorting motifs were identified in spruce POXs PX1, PX2 and PX3. However, multiple sequence alignments with HRPC and other POX sequences showed that two of the Norway spruce POXs, PX2 and PX3, contained C-terminal extensions (CPs) after the terminal asparagine typical for the majority of POXs (III, IV). Such extensions are not found from the other full-length POXs isolated from Norway spruce (Fossdal et al. 2001, Koutaniemi et al. 2005, Warinowsky et al. unpublished). It has been suggested that these extensions are ctVSDs in POXs (Theilade et al. 1993). In fact, Matsui et al. (2003) confirmed the VSD function of CP in HRPC by expressing fusion genes coding for green fluorescent protein (GFP) and N-terminal SS and CP peptides from HRPC in tobacco cells. Furthermore, Liu et al. (2005) demonstrated that CP in POX TmPRX8 from diploid wheat (*Triticum monococcum*) was sufficient to direct GFP into the vacuole even in the absence of an N-terminal SS.

For studying the function of the putative N-terminal SSs and ctVSDs in Norway spruce POXs PX1, PX2 and PX3, fusion genes coding for enhanced green fluorescent protein (EGFP) and N-terminal and C-terminal fragments from spruce POXs were prepared (III, IV). The fusion genes were transiently expressed in tobacco leaf mesophyll protoplasts under the 35S promoter. EGFP fluorescence distribution in cells was compared with EGFP without any signal sequences (cytosolic control) and with EGFP fused to verified SS from tobacco chitinase (III).

Tobacco leaf mesophyll protoplasts have been used earlier for studying the localization of for example chitinase (DiSansebastiano et al. 1998), phaseolin (Park et al. 2004) and galactan-galactan galactosyltransferases (GGT) (Tapernoux-Lüthi et al. 2007). Here, protoplasts were prepared from tobacco (*Nicotiana tabacum*) plants grown in greenhouse

or *in vitro* conditions and transformed either by electroporation or PEG-mediated gene transfer (III, IV). Best yields and transformation frequencies (up to approximately 20%) were obtained when using young *in vitro* plants and PEG-mediated gene transfer, possibly due to thinner cuticles in leaves and avoidance of stressing surface sterilization steps (IV).

EGFP fluorescence was observed in tobacco protoplasts with a confocal microscope, which enables high resolution imaging even in relatively thick samples (here around 40 µm) and distinction between EGFP fluorescence and autofluoresence arising from chloroplasts (III, IV). The most difficult aspect in using fluorescent imaging of EGFP in tobacco protoplasts appears when EGFP is located to the central vacuole, where fluorescence is often weak or diffuse, and can thus be confused with green autofluoresence emitted by the central vacuole of some non-transformed cells (IV).

Confocal images from protoplasts expressing N-terminal fragments from PX1, PX2 and PX3 fused to the N-terminus of EGFP were characterized by ER network-like localization of EGFP (III, Figure 5A-C), showing that the N-terminal peptides in spruce POXs are functional SSs. Typically, EGFP fluorescence was also observed in the nuclear envelope, in contrast to cytosolic EGFP which accumulated inside the nucleus (III, Figure 5D). Similar EGFP fluorescence distributions have been observed with expression of EGFP fusions of N-terminal SSs of tobacco chitinase (DiSansebastiano et al. 1998).

In tobacco protoplasts expressing EGFP fused with N-terminal and C-terminal peptides from PX2 and PX3, localization was different from that of the merely N-terminal SS directed one. In tobacco protoplasts expressing fusion genes of PX2 N-terminal SS- and CP-peptides, EGFP fluorescence was observed in medium-sized globular and small punctate or tubular structures (IV, Figure 5 E, F). The medium sized globular structures resembled the small vacuoles in tobacco cells observed by Matsui et al. (2003) in a study on the HRPC localization, whereas the punctate structures resembled Golgi bodies or pre-vacuolar compartments involved in post-ER trafficking to the vacuoles (Hanton and Brandizzi 2006).

In tobacco protoplasts transformed with fusion genes of N-terminal secretion signal and putative ctVSD from PX3 and EGFP, green fluorescence was observed in the first days of culture in relatively large sheet-like or tubular structures (IV, Figure 5 G, H). However, at the later phases of culture the most frequent green fluorescence pattern was ER-network like, as observed in the protoplasts expressing fusions of EGFP and SSs peptide only (IV, Figure 5 I). ER-related sheet-like structures have been observed in plants (Hawes et al. 2001), and it may be possible that some of the sheet-like structures observed in tobacco protoplasts expressing fusions of EGFP and SSs and CPs from PX3 are part of the ER-membrane network (IV).

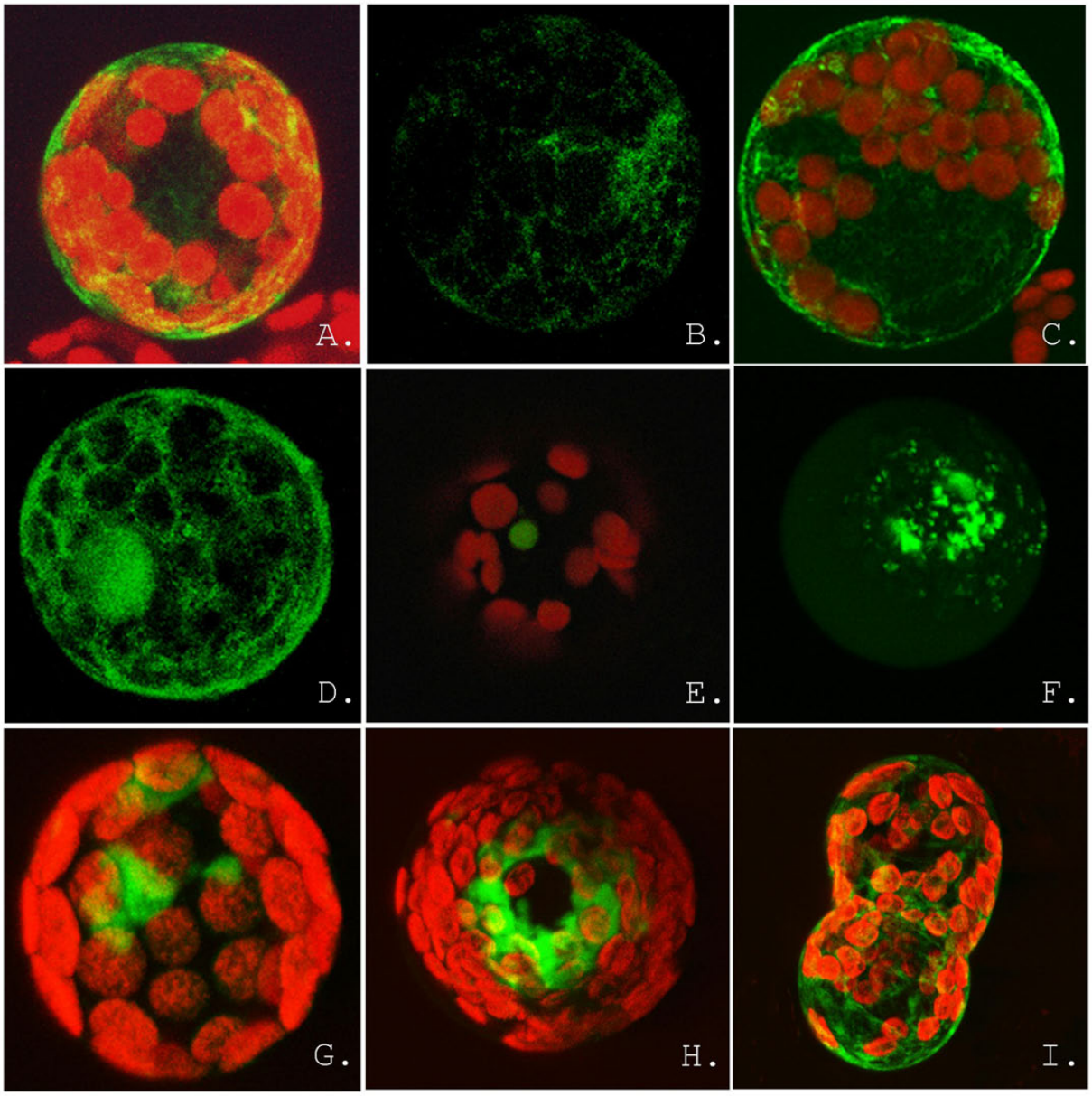


Figure 5. Tobacco protoplasts expressing fusion genes of *egfp* and 5' and 3' fragments of Norway spruce POXs *px1*, *px2* and *px3* under the 35S promoter. Red represents clorophyl autofluorescence, subtracted from the images B, D and F. Fusion genes used in transformation were coding for PX1 SS-EGFP-HDEL (ER-retention motif for signal enhancement) (A), PX2 SS-EGFP-HDEL (B), PX3 SS-EGFP-HDEL (C), cytosolic EGFP (control)(D), PX2 SS-EGFP-CP (E and F, 2d and 5d after transformation) or PX3 SS-EGFP-CP (F and H, 2d after transformation; I, 7d after transformation).

Structural variation in CPs in POXs was studied in POX sequences from *Arabidopsis thaliana*, rice (*Oryza sativa*), Western Balsam poplar (*Populus trichocarpa*) and all the gymnosperm POXs found in the Peroxibase (IV). The C-terminal sequences after the conserved asparagine (N334 in HRPC) were aligned in each species/plant group and compared with functionally verified CPs from HRPC and TmPRX8 (IV).

Since the non-sequence specific vacuolar sorting signals are typically rich in hydrophobic amino acids, grand average hydropathicity (GRAVY, Kyte and Doolittle, 1982) values were calculated for the CPs in order to reveal the level of hydrophobicity of the peptides (if positive, hydrophobic surroundings are expected) (IV). In most of the CPs from dicotyledon plants, the GRAVY-values were positive, whereas in rice, for only half of the

CPs the GRAVY-values were positive. In the CPs from gymnosperm POXs, the only POX with a CP with a positive GRAVY-value in this study was PX2.

Five of the CPs in POX from *A. thaliana* showed remarkable similarity to the functionally verified CP in HRPC, with positive GRAVY-values, terminal motif FV/ASS/FM and repeating doublets of hydrophobic amino acids (IV). In a proteomic analysis of vacuoles in *A. thaliana* leaves, all these POXs were found from the vegetative (lytic) vacuole (Carter et al. 2004). Interestingly, the CP in PX2, the only one with a positive GRAVY-value among the gymnosperm CPs, showed similar terminal structure to the CP in vacuolar *A. thaliana* POX AtPRX38 (IV).

The second frequently observed extension type in POXs abundant in poplar POXs had also positive GRAVY values and contained terminal -VSSI motif. Similar extensions were also observed in two of the *A. thaliana* POXs, but with negative GRAVY-values, due to highly hydrophilic amino acid residues in the middle of the peptide (IV). Most variant CP structures both in length and in sequence were observed in rice POXs. The most frequent amino acid in these extensions was alanine, which occurred in doublets, triplets and quartlets alone or in combination with other hydrophobic amino acids (IV). The greatest structural similarity to functionally verified CP from TmPRX8 was observed with OsPRX15 from rice, characterized by an S-repeat near the beginning of the CP (IV).

Two types of CPs were most frequently observed in gymnosperm POXs. PX3, PSYP1 and a group of other POXs from *Pinus* species contained almost identical CPs of 18 amino acids with the terminal motif -SYSM and negative GRAVY values (IV). In the other group containing extensions from two *Pinus* species and Sitka spruce (*Picea sitchensis*), CPs were only seven amino acids long, had very low GRAVY-values and had also almost identical sequence (IV).

According to the the three dimensional structural models from spruce POXs, both N-terminal and C-terminal regions of the protein chains are located to the surface of PX1-3 thus being available for recognition of protein sorting systems (IV). Apparently, proteins can enter in the vacuoles at least via Golgi and the pre-vacuolar compartment, or directly from ER, the latter route being possibly used for proteins in which Golgi modifications are not needed for protein maturation (reviewed by Robinson et al. 2005). BP-80-family receptors are involved at least in the recognition of sequence specific VSDs and subsequent localization of the proteins into lytic vacuoles. Receptors mediating protein sorting into storage vacuole have been identified for ctVSD-containing storage proteins in *A. thaliana* and pumpkin (Shimada et al. 2002, 2003). The binding of ctVSD to these receptors is Ca^{2+}-dependent, but the interactions between the receptors and the amino acids in the ctVSD are not kown.

According to this study, the PX1 is a cell wall protein, whereas PX2 is directed to small vacuole-like structures by ctVSD. In contrast to PX2, CP in PX3 did not function in a similar way in tobacco cells, suggesting for cell wall localization for this POX.

3.5. Conclusions

In this work, participation of POXs in the polymerization of lignin in secondary xylem of Norway spruce (*Picea abies*), Scots pine (*Pinus sylvestris*) and silver birch (*Betula pendula*) was investigated by measuring the xylem POX activities through the growing season and studying the ability of the xylem POXs to oxidaze different monolignol substrates. In addition, three *pox* cDNAs were cloned from lignifying xylem of Norway spruce. Structures of the translated protein products of these novel spruce *pox*s were compared to other known POXs, their cell-specific expression was studied by *in situ* hybridization and their subcellular localization was determined by transient expression of fusions of EGFP and spruce POX N-terminal and C-terminal peptides in tobacco protoplasts.

Elevated POX activities were seen in Norway spruce, Scots pine and silver birch

xylem samples at the time of secondary growth and lignification, but also in late autumn and winter after cessation of growth. POX activities in tree trunks has not been studied to this extent elsewhere. It is interesting that although POX activity patterns varied in xylem of different tree species during growth, they were in general strongly increased in autumn, suggesting that they are involved in common physiological process occurring in all tree species studied here.

The POX activities in xylem of Norway spruce, Scots pine and silver birch originated from multiple monolignol-oxidizing POX isoforms, most of which prefered coniferyl alcohol as a substrate. However, syringyl-oxidizing POX isoforms were found here in the xylem of both Norway spruce and silver birch irrespective of their lignin chemistry. This is interesting, especially considering putative participation of syringyl-peroxidases in the oxidation of lignin polymers.

Cloning and characterization of the three Norway spruce *pox* cDNAs, *px1*, *px2* and *px3* has given new information on structural variation of POXs gymnosperm species. The translated protein sequences of three spruce POX cDNAs studied here were less than 60% identical to each other and they fell into different phylogenetic groups. The most cationic PX1 showed highest similarity to lignin-binding POXs from Norway spruce tissue culture, whereas PX2 and PX3 clustered with various POXs from pine species.

One of the Norway spruce *pox*s studied here *px1* has the required characteristics for the involvement in lignification of the secondary cell walls of spruce tracheids: it is expressed in lignifying tracheids, it codes for a cationic enzyme similar to monolignol oxidizing POXs found in lignifying xylem of Norway spruce and it has a functional SS-peptide for secretion into the cell wall. As *px1*, *px2* is expressed in developing spruce tracheids, but its expression is also induced by compression stress and fungal infection (Koutaniemi et al. 2007). In addition, C-terminal peptide in PX2 protein acts as a vacuolar localization signal in tobacco cells. However, it is also possible that the site of action for different POXs is influenced by the type or state of the cell, for example in developing xylem the originally vacuole located POXs may participate the last stages of cell wall production after the rupture of the vacuole during programmed cell death. *Px3* mRNA was not detected in developing tracheids of spruce seedlings, but the experiments with tobacco protoplasts suggest that the cell wall localization for PX3 protein must be considered irrespective of the CP in this POX. Phylogenetic relative of PX3, PSYP1, was found from short-roots of *Pinus sylvestris* and has an almost identical CP with PX3 (Tarkka et al. 2000). The authors suggested that PSYP1 is related to reduced cell elongation (Tarkka et al. 2000). However, similarly to *px2*, *px3* gene expression is induced by fungal infection in Norway spruce (Koutaniemi et al. 2007) and thus PX3 protein may be related to defence responses in the apoplastic space.

Transgenic approach including over-expression and down-regulation of *px1*, *px2* and *px3* in Norway spruce trees has been initiated for revealing their ability to modify wood properties. However, due to the high amount of coniferyl alcohol oxidizing POXs seen in the developing xylem of Norway spruce, genetic modification of expression of multiple *poxs* simultaneously may be needed to achieve significant changes. Production of *px1*, *px2*, *px3* and other POXs from trees by heterologous expression and studying of their catalytic properties, possible regulation by post-translational modifications and interactions with cell wall components, would further clarify functional determinants of POXs in trees. In future, the identification and structural characterization of syringyl-oxidizing POXs observed here in both conifers and angiosperm trees would be of special interest. Finding of the structural properties that allow oxidation of sinapyl-compounds and possibly polymeric lignin by these POXs may give new criteria for the search of lignin modifying POXs.

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Errata

3. Results and Discussion, 3.4 Subcellular localization of POXs
Page 35, right column, line 13: …observed by Di Sansebastiano et al. (1998) in study of tobacco chitinase localization.

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